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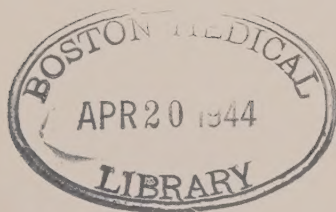
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THE DIFFERENTIATION AND SIGNIFICANCE OF ARGENTAFFIN GRANULES IN THE HYPOPHYSIS ¹

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FIVE FIGURES

In addition to the three classical types of cells of the pars anterior of the hypophysis, the alpha, the beta and the chief cells, several variants have been described in recent years. Among those mentioned occasionally in the literature are the silver reducing cells. However, virtually nothing is known about the histogenesis of these cells. It has seemed desirable, therefore, to apply newly devised methods of silver impregnation and differential staining to the study of this problem.

MATERIAL AND METHODS

The material consisted of hypophyses of rabbits and man. Most of the rabbits had been used for ovulation tests. Thirty-six of the rabbits gave positive and forty-five negative tests. The controls consisted of ten virgin rabbits and of ten male rabbits. Six female rabbits received an intravenous injection of 320 mg. of cobaltous chloride ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) in 10 cc. of 4% aqueous solution of sodium citrate and were killed 1 hour after injection. Two female rabbits received intravenous injection of 10 cc. of 10% sodium bicarbonate and were killed after 1 hour. Two female rabbits received intravenous injection of 20 cc. of distilled water and were killed $\frac{1}{2}$ hour later. Four female rabbits received subcutaneous injections of 0.25 gm. sulfa-

¹ This work has been aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

pyridine per kilo weight every other day in total amounts of 1 gm., 1.5 gm., 2 gm., and 2.5 gm. and were killed 48 hours after last injection. Four female rabbits each received two intravenous injections of 1 cc. of 24-hour broth cultures of streptococcus hemolyticus on 2 successive days and were killed on the third day.

The animals were kept in sunny, airy quarters, fed a well-balanced diet with an abundance of fresh greens. The investigations were carried out during the whole year in order to evaluate possible seasonal variations. The animals were killed in uniform fashion by air embolism. Following the removal the hypophysis was fixed either whole or after being cut in two in a sagittal plane.

The human material consisted of hypophyses from fourteen adults and eight infants. With the exception of two surgical cases, the human material was obtained post-mortem; each hypophysis was cut in several parts and placed in the fixative within $\frac{1}{2}$ hour to 3 hours after death.

Parallel studies were made with the use of the following three methods of fixation. In the first method tissue was fixed for 24 hours in Helly's fluid. After washing in distilled water for 24 hours tissue was dehydrated as usual and imbedded in paraffin. In the second method tissue was fixed for 24 hours in a solution consisting of 20% formol and 1% of Gröblers water soluble safranin in equal parts. After rinsing in distilled water, tissue was placed for 24 hours in Helly's fluid and thereafter it was treated in usual manner. In the third method tissue was fixed for 24 hours in a solution consisting of 45% formol, 1.5% of safranin and 4% acetic acid in equal parts. After rinsing in distilled water tissue was placed for 24 hours in Zenker's solution and thereafter was treated in the usual manner. The rationale of the last two methods of fixation is discussed elsewhere by N. W. Popoff ('43). Tissue was cut into serial sections 5 μ thick and mounted upon slides with the aid of the gelatin-formol technic of Masson ('23). Following fixation with first and third fixatives, slides were stained with potassium bichromate-eosin-silver-methyl blue method. The

material fixed with the second fixative was stained with the safranin-silver-methyl blue method. Both staining methods were devised by N. W. Popoff ('39). A slight modification was introduced in the technic of staining and it involved the preparation of the reducer: the Grüber's Wasserblau could not be secured and was replaced with Grüber's methyl blue (H 164) and was used in 1% solution. In the preparation of the reducer 1 drop of hydrazine hydrate was added to 2 cc. of methyl blue.

OBSERVATIONS

The staining of the material fixed in Helly's fluid with the potassium bichromate-eosin-silver-methyl blue method offers great advantages for the study of the chromophil cells and it was therefore used most extensively in these investigations. In the sections stained with this method the alpha cells take eosin stain and the beta cells the methyl blue. The terms acidophilic and basophilic cells could not be employed since both of the dyes used are acid dyes. The agranular alpha cells appear light pink and the granules of the granular alpha cells are red, orange or rusty brown. The beta cells stain more or less deeply with the methyl blue and the chief cells take a very faint greenish stain. The impregnation of the reticulum with this method is perfect and this facilitates the study of topographic distribution of various cellular constituents of the hypophysis. The nuclei of the chromophil cells are vesicular or they appear dense, homogeneous and are stained with eosin or methyl blue. In rare instances they show a faint silver reduction. Clear, vesicular nuclei are found most commonly in the agranular alpha cells, while the alpha cells with distinct granulations show, more frequently, homogeneous dull blue nuclei.

In close association with alpha cells there are cells which appear round or pear-shaped and their cytoplasm is densely packed with small, uniform argentaffin granules (fig. 1).² They occupy the central portion of the hypophysis and are found at the periphery of the alveolus and in close proximity to the

² Camera lucida drawings were made by Dr. Nicholas Popoff.

blood vessels. The nuclei of these argentaffin cells are clear and show no stainable chromatin network or nucleolar membrane (fig. 2). Side by side with the typical jet black argentaffin cells there are granular alpha cells, which contain rusty or brown granules (fig. 2). Furthermore, argentaffin cells may be observed which show a gradual decrease in the number of

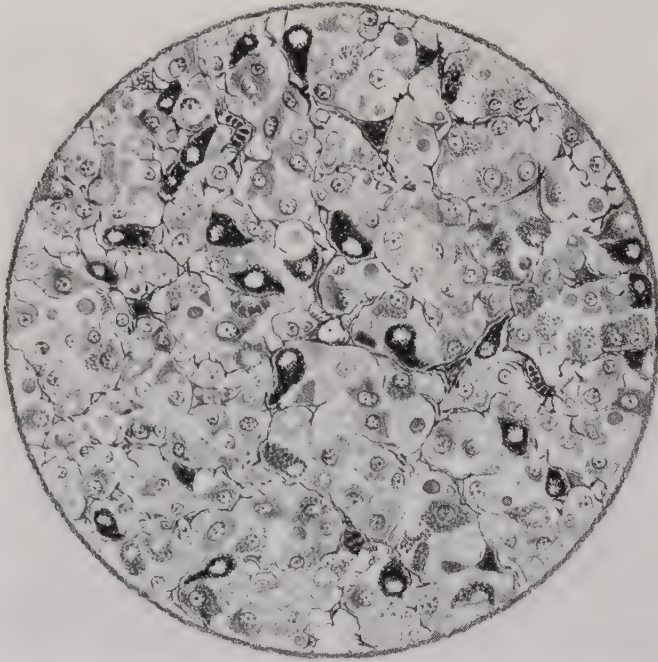


Fig. 1 Camera lucida drawing of the rabbit's hypophysis showing general distribution and appearance of the argentaffin cells. $\times 250$.

the granules and in the intensity of silver reduction. Such cells contain only a few brownish or yellowish granules, their nuclei acquire vesicular character, they diminish in size and finally become indistinguishable from the chief cells. The sum of observations then indicates, that the alpha cells pass through a specific cytomorphosis which leads to the formation of the argentaffin cells; these, in course of further metamorphosis, lose their granules and become chief cells. A similar

process of transformation of beta cells into argentaftin cells with their consequent return to chief cells has been observed in the human hypophysis but rarely in rabbits.

On the periphery of the alveoli in close proximity to the reticulum there are often found small, shrunken triangular or

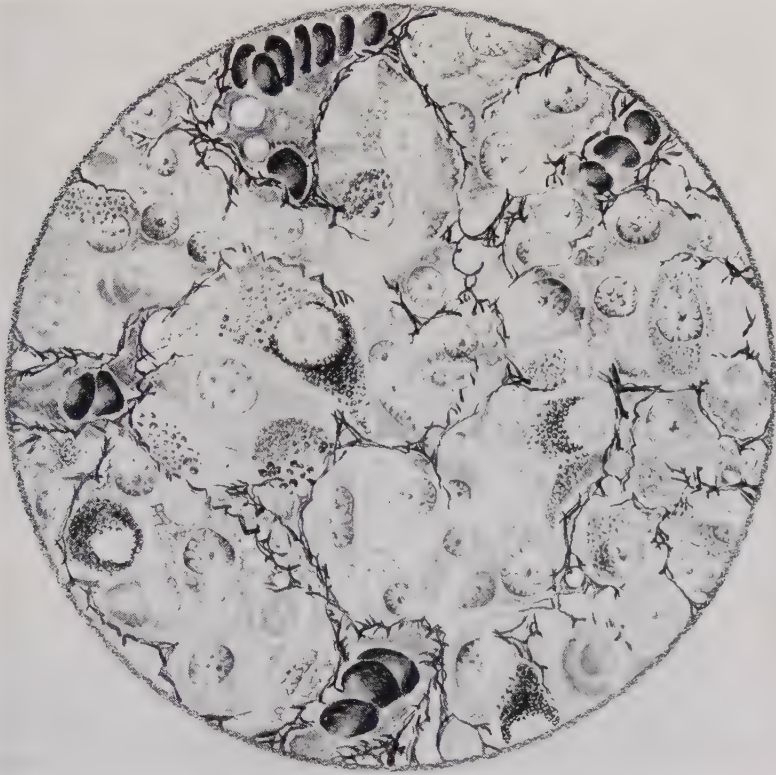


Fig. 2 Camera lucida drawing of the rabbit's hypophysis showing typical argentaftin cells and intermediate forms between granular alpha cells and argentaftin cells. Note the relation of the argentaftin cells to the reticulum. $\times 870$.

elongated cells with homogeneous dark red or blue cytoplasm and condensed blue or red nuclei. These cells are apparently related to the small-sized chromophils with intensely stained nucleus and protoplasm.

Studies of the hypophyses of rabbits subjected to intravenous injections of cobaltous chloride ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$), of sodium

bicarbonate, of distilled water and of cultures of hemolytic streptococci and to subcutaneous injections of sulfapyridin have shown, that the above procedures failed to affect the appearance and number of the argentaffin cells. Ovulation induced in the rabbits by injection of urine from pregnant women failed to influence the argentaffin cells. The effect of chemicals upon the argentaffin cells was tested as follows: unstained mounted sections were treated with (a) normal hydrochloric acid (pH 0.1 for 1 hour at 60° C.); (b) N/1000 sodium hydroxide (pH 11, for 12 hours at 37° C.); (c) 3% hydrogen peroxide for 24 hours at 37° C. No qualitative or quantitative changes were observed in the argentaffin granules in the sections treated with above chemical substances and impregnated afterwards with silver nitrate. In sections treated with nitric acid followed with liquid ammonia, the granules gave a positive murexide reaction, which indicates, that they contain a purine compound. Freezing of the hypophysis by means of CO₂ for 10 minutes preliminarily to the fixation did not change the silver reducing qualities of the granules. It is worthy of mentioning, however, that freezing destroys the selective tinctorial affinity of the chromophil cells; after this procedure the alpha cells fail completely to take eosin and the beta cells take methyl blue in a defective and indistinct manner. Heating of the gland for 1 hour in water at 90° C. did not affect the silver reducing properties of the argentaffin cells nor the tinctorial selective affinity of other cells.

Present investigations were carried throughout an entire year and it is most significant, that during the months of December and January there was a considerable decrease in the number of the argentaffin cells in the hypophyses of the rabbits. Moreover, no argentaffin cells were found in the hypophyses of some animals examined during this season.

In material fixed in a solution of formol-safranin followed by Helly's fluid (fixative 2), and stained with safranin-silver-methyl blue, there are found in addition to the typical argentaffin granules, delicate silver reducing granules and rods uni-

formly distributed in the cellular constituents of the hypophysis. These cytoplasmic inclusions correspond in every way to typical mitochondria and are easily distinguishable from the argentaffin granules. This difference is well shown in the hypophyses heated for 1 hour at 90° C., prior to fixation. This procedure destroys the silver reducing properties of the mitochondria without affecting the silver reducing properties of the argentaffin cells.

In the hypophyses fixed in formol-safranin-acetic acid mixture followed with Zenker's fluid (fixative 3) and stained with potassium bichromate-eosin-silver-methyl blue, there are silver reducing granules which differ from the granules so far described. Sections thus treated show numerous angular bulky granules and they are found in the alpha cells, the beta cells, the vascular endothelium, the pericytes and occasionally in the lumen of the capillaries (fig. 3). No granules of this character were found in material fixed in Helly's fluid. It is significant moreover, that these granules were not found in the material examined after a delayed fixation of 24 hours, or in the sections immersed overnight in methyl alcohol. It should be noted, that animals used in these investigations were kept on a high vitamin-C diet and that the findings of the granules in the hypophyses were constant and uniform. When the above facts are taken into consideration together with the chemical nature of the fixative used and the topographic distribution of the granules, little doubt remains that the granules in question are of the nature of the ascorbic acid. The well known high content of the ascorbic acid in the hypophysis (Giroud et Leblond, '34) serves as additional evidence in support of this conclusion.

In the hypophyses of the newborn, of infants and young adults the argentaffin cells are numerous and exhibit topographic and cytologic characteristics similar to those reported in rabbits (fig. 4). In the hypophyses of the aged the argentaffin cells are rare. No argentaffin cells were found in the two chromophobic adenomas available for examination.

In the human hypophyses fixed with formol-safranin-acetic acid mixture followed with Zenker's fluid (fixative 3) and stained with potassium bichromate-eosin-silver-methyl blue, there were also found silver reducing granules of the nature

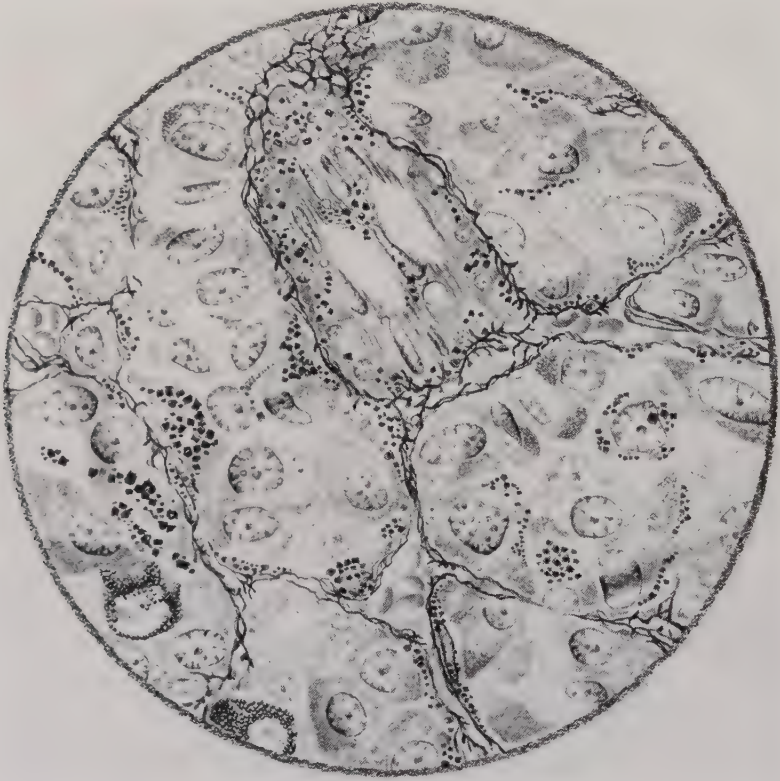


Fig. 3 Camera lucida drawing of the rabbit's hypophysis showing the bulky angular silver reducing granules (ascorbic acid) in the chromophil cells, vascular endothelium, pericytes and vascular spaces. Typical argentaffin cells with granules of different kind are also shown. $\times 870$.

of the ascorbic acid. The number and the distribution of these granules varies and is less constant than it is in the rabbits. In the newborn these granules were quite numerous but they were rare in adults who died after protracted illness and they were not found in aged people with obvious signs of emaciation and senile debility.

An additional feature of cytological importance is the demonstration of specific silver reducing inclusions in the beta cells of human hypophysis. These inclusions measure from a fraction of 1 micron to 4 microns and are seen in material that has been fixed in Helly's fluid and in other fixatives and stained

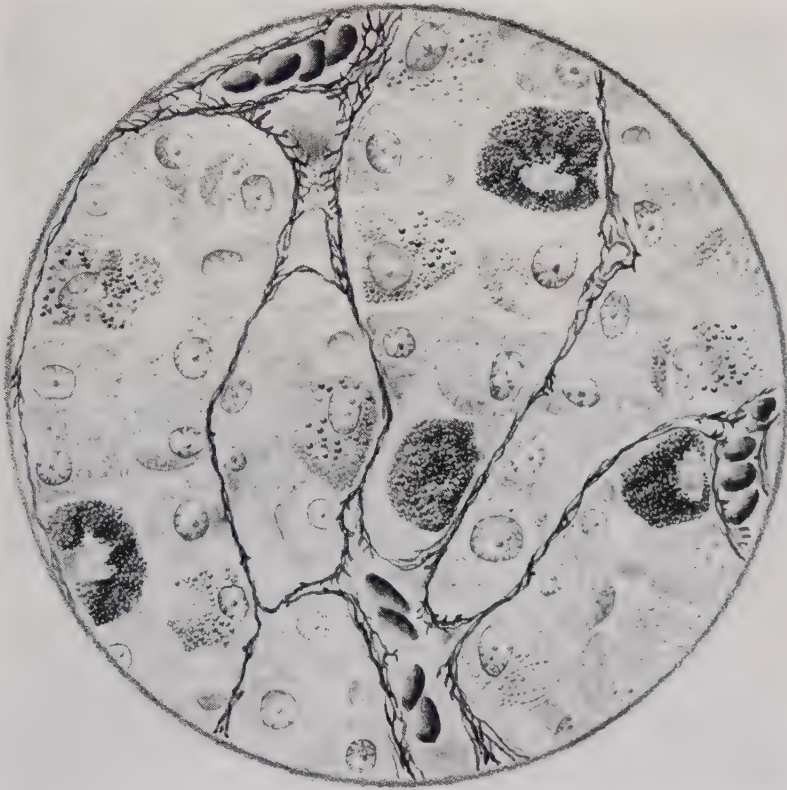


Fig. 4 Camera lucida drawing of infant hypophysis showing argentaffin cells and their relation to the reticulum and cellular constituents. $\times 870$.

with potassium bichromate-eosin-silver-methyl blue. Together with these inclusions there are found yellow refractive vacuoles bordered with numerous coarse silver reducing granules (fig. 5). These inclusions are scarce in young people but are extremely numerous in aged people. The appearance and

distribution of these inclusions and their association with refractive vacuoles favor the assumption that these inclusions are of lipoid nature. It should be noted that no inclusions of similar character were found in the argentaffin cells.

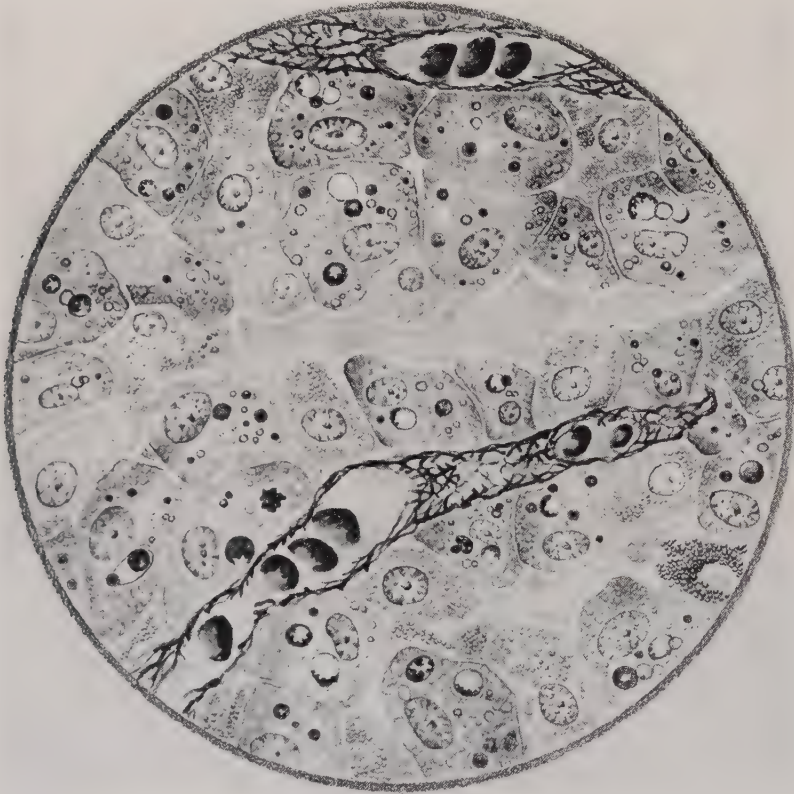


Fig. 5 Camera lucida drawing of the hypophysis of a middle-aged human showing beta cells containing vacuoles and silver reducing inclusions of apparently lipoid nature. Note the absence of similar inclusions in alpha and argentaffin cells. $\times 870$.

DISCUSSION

The main histological methods employed up to date for the study of the hypophysis permit a satisfactory tinctorial differentiation of cellular constituents of the gland but they are not fully adequate for the studies of intracellular inclusions. Most

helpful among these methods are those of Severinghaus ('32); Dawson and Friedgood ('38); Dawson ('39) and Maxwell ('38). The technic used in these studies combines selective staining with silver impregnation and thus permits more precise histological differentiation of the intracellular inclusions. Furthermore the variants of this technic are helpful in defining the nature of individual intracellular inclusions which possess silver reducing properties.

In regard to the typical argentaffin granules two processes of cellular metamorphosis were observed: one leading to the formation of argentaffin cell from the chromophil cell and the other showing the transformation of the argentaffin cell into the chromophobe cell. The transformation of chromophils into the chromophobes through loss of specific granules has been reported by Severinghaus ('33, '37) and Bailiff ('38). This is in agreement with the results of present investigations. All phases of such transformation have been traced with the argentaffin cells representing an intermediate link between the two types of cells. The established fact is that the typical argentaffin granules are not found outside the cells and that the elimination of the argentaffin cells by phagocytosis or by any other means was never observed. In not a single instance have mitoses been observed in argentaffin cells. It is generally accepted that in the hypophysis of normal animals mitotic figures are infrequent. It has been pointed out by Baer ('40), that the number of mitoses in certain type of cells does not correspond to the relative frequency of these cells and, therefore, that the formation of characteristic types of cells must be achieved by some other means.

From the data reported it is apparent, that one of the chief characteristics of the discussed argentaffin granules is their resistance to the action of certain physical and chemical agents. In this respect they resemble closely granules of the argentaffin cells of the intestines (Popoff, N. W. '39; Jacobson '39). The chemical nature of argentaffin granules of the intestinal cells has been discussed by a number of investigators. Lison ('36) from the evidence furnished by his own work and by the

work of others, concluded that the argentaffin granules contain an orthodioxymethyl in para-position. Jacobson's ('39) excellent contribution to this subject suggests strongly that the argentaffin granules of the gastrointestinal tract contain a xanthopterin or uropterin and that these substances, in his opinion, are actually responsible for the silver reduction reaction. They are related to the metabolism of purine and are characterized by remarkable resistance to the action of chemical and physical agents. Taking into consideration the discussed similarity between the argentaffin granules of the hypophysis and those of the intestinal tract it is reasonable to assume, that the argentaffin granules of the hypophysis contain also a substance of pterin nature.

Neither functional longevity of the cells of the hypophysis nor the question of replacement of functionally worn-out cells have been elucidated by previous investigators. These studies indicate that there exist in the functional life of chromophil cells alternating periods of rest and activity. The functionally exhausted chromophil cell passes apparently through the phase of cellular cytomorphosis and is gradually transformed into the argentaffin cell which thus represents a phase of rest or recuperation. Such interpretation is in accord with the biological concept of epithelial functional rejuvenation presented by Popoff, N. W. ('39) in his publication on the significance of argentaffin cells in the gastrointestinal tract. It must be mentioned that in the few reports dealing with the argentaffin cells of the hypophysis hormone producing qualities are attributed to these cells. Eros ('28) for instance, in his work on the argentaffin cells of the stomach and intestines discusses briefly the argentaffin cells of the hypophysis and he attributes to them an endocrine function. His assumption is based solely on the analogy between argentaffin cells of the gastrointestinal tract and those of hypophysis. Morita ('37) has observed in the pregnant animals an increase in the number and size of the argentaffin cells of the hypophysis and an intensification of silver reduction and he concludes that these cells play a part in hormone production. However, no sufficient corrobora-

tive evidence in support of this concept is offered by this author.

In these studies particular attention has been given to the differentiation of argentaffin granules from other cytoplasmic inclusions and it has been definitely established that argentaffin granules are unrelated to the mitochondria.

The object of these studies was to define and to differentiate various intracellular silver reducing inclusions of the pituitary cells. The application of special acid fixative has disclosed the granules which are fundamentally different from the typical argentaffin granules. These granules display all characteristics of ascorbic acid, a constituent of the normal gland, demonstrated with the aid of silver reduction method by Giroud and Leblond ('34).

In the material fixed in Helly's fluid and stained with potassium bichromate-eosin-silver-methyl blue there were found in the beta cells in the hypophysis of man silver reducing inclusions. These inclusions differ in every way from typical argentaffin granules and they are apparently of lipid nature. This conclusion finds support in the studies of Kraus ('12) who believes that the accumulation of lipoids is an expression of the physiologic phenomenon of aging. Our results are in full agreement with his concept.

This discussion would be incomplete without mentioning the matter of seasonal variations in the number of typical argentaffin cells in the hypophysis of rabbit. It has been found that during the months of December and January the argentaffin cells were rare or were not found at all. This marked reduction in the number of the argentaffin cells together with an increase in the number of the small agranular cells could signify in the light of the above discussion, a diminished activity of the gland. Such interpretation is in agreement with the recent observations by Friedman and Friedman ('39). Unexpectedly these authors have found definite variations in the hormonal content of the hypophysis of the rabbit, the lowest level being reached in early January.

SUMMARY

1. New histologic methods which combine selective staining and silver impregnation were applied to studies of the hypophyses of man and rabbits.
2. These methods permit demonstration and clear cut differentiation of the following silver reducing inclusions in the cells of the anterior part of the hypophysis: (a) granules of typical argentaffin cells, (b) mitochondria, (c) ascorbic acid, and (d) certain inclusions of lipid nature.
3. Two processes of cellular metamorphosis were described: one leading to the formation of argentaffin cells from chromophil cells, the other demonstrating the transformation of argentaffin cells into chromophobe cells.
4. The conclusion was reached that the argentaffin cell represents in the life cycle of the chromophil cell a phase of rest or recuperation and that the biological concept of epithelial functional rejuvenation is applicable to the hypophysis.
5. In their mode of formation and in their response to various chemical and physical agents the argentaffin cells of the hypophysis are analogous to those of the gastrointestinal tract.
6. The seasonal variations in the histologic pattern of the hypophysis of the rabbit were studied and a striking decrease in the number of argentaffin cells was noted in mid-winter.

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THE FATE OF THE UNFERTILIZED OVA IN THE ALBINO RAT ¹

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ONE PLATE (SIX FIGURES)

If ovulation occurs without fertilization, the liberated ova undergo rapid devitalization in the Fallopian tubes and uterus. The vitellus degenerates and undergoes fragmentation. Evidence for this statement has been obtained by observations on many different animals (Hartman, '24; Smith, '25; in the opossum; Sobotta, 1895; Kirkman, '07; Long, '12; Charlton, '17; in the mouse; Long and Evans, '22; Mann, '24; Blandau and Jordan, '41; in the rat; Squier, '32; Blandau and Young, '39; in the guinea pig; Heape, '05; Pincus, '36; in the rabbit; Hartman, Lewis, Miller and Swett, '31; in the cow; and Allen, Pratt, Newell and Bland, '30; in man).

The ultimate fate of these degenerating ova is, nevertheless, a problem which has not been satisfactorily resolved for any mammal. The general assumption has been that the degenerating eggs undergo dissolution and are resorbed in either the tubes of the uterus (Pincus, '30 and '36; Corner, '28). It is also generally believed that unfertilized ova disappear completely before the succeeding ovulation, the only contradictory report being that of Hensen (1869) who describes the retention of approximately 100 rabbit ova in a blocked oviduct in which the ova had presumably accumulated from a number of ovulations. It has been stated that final disintegration of unfertilized ova in the mouse is affected by means of phagocytic leucocytes (Charlton, '17).

¹ This investigation was supported by a grant from the Committee for Research in Problems of Sex, National Research Council.

During the period of sexual receptivity in the rat the uterine cornua become distended to unusual proportions with a clear, watery fluid (Long and Evans, '22; Astwood, '39). While examining this fluid in a routine manner during the early hours of heat, the present investigator discovered degenerating ova in it. This accidental finding became the basis for the present investigation. In extending this observation we sought to determine: (1) the number of degenerating ova that can be recovered from the reproductive passages of the female rat within 1 to 12 hours after the onset of heat; (2) whether ova from more than one ovulation can be recovered from the reproductive tract during a single reproductive cycle; and (3) the span of time during which ova can be recovered from the reproductive passages in the pseudopregnant rat.

MATERIALS AND METHODS

Washings of the Fallopian tubes, uterine cornua and vaginae of seventy mature female albino rats were examined at varying intervals during and following sexual receptivity. Twenty females were killed from 1 to 4 hours and seventeen females from 8 to 12 hours after the beginning of heat. The animals were killed by decapitation and the entire reproductive tract carefully removed. The vagina was severed at the cervix, placed in a separate receptacle and thoroughly washed in warm Locke's solution. The fluid was carefully searched for the presence of eggs. The uterine cornua were flushed with warmed Locke's solution and the collected fluids were examined for ova. The coiled oviducts were placed under a dissecting microscope and straightened by trimming away the mesosalpinx with a pair of iridectomy scissors. The content of the tubes was expressed by stroking their lengths with a curved probe. The ova recovered from any portion of the reproductive tract were easily identified because of the highly translucent zona pellucida. The ova which were to be photographed were removed to a clean cover slip by means of a fine pipette. The cover slip was then sealed to a hanging drop slide by vaseline and placed on an automatic stage warmer,

Thirty degenerating ova, obtained from the uterine cornua of five animals, were mounted on slides and stained in toto with a nuclear stain to determine the presence or absence of phagocytic leucocytes within them.

Pseudopregnancy was induced in thirty-three animals by electrical stimulation of the cervixes of females in heat (Greep and Hisaw, '38). At the appropriate times the reproductive tracts were removed and examined as described above.

TABLE 1

Number of degenerating uterine ova recovered from animals killed 1 to 4 hours after the beginning of heat.

NUMBER OF EGGS	NUMBER OF ANIMALS
4	1
5	2
6	3
7	3
8	7
9	2
10	2
—	—
Total 49	20

OBSERVATIONS AND DISCUSSION

Approximately 96 hours after a preceding ovulation and 4 to 5 hours previous to an impending one (i. e., 1-4 hours after the onset of heat) degenerating ova were invariably recovered from the fluids of the uterine cornua (table 1), and none were found in the oviducts or vagina. Four to ten ova (average 7.4) were obtained from the twenty females examined. The average number of ova recovered is somewhat less than that expected on the basis of litter size. This discrepancy may be accounted for by the difficulty of locating all of the ova in the large amount of uterine fluid and debris.

Figures 1 and 2 are photographs of ova recovered from the uterine fluids 1 to 4 hours after the beginning of heat. Around each egg an intact zona pellucida is visible. In the majority the

cytoplasm and nucleus have fragmented into numerous conspicuous vesicles and punctate granules which vary in size.

The step by step process of degeneration and fragmentation of the ovum has been described by Charlton ('17) for the mouse, Mann ('24) for the rat, and Smith ('25) for the opossum. It is sufficient to indicate that there is great variability in the extent of fragmentation in the ova recovered from a single animal. At this period the eggs are no longer spherical but disc-shaped and may or may not be free from adherent cells, debris and leucocytes.

As pointed out above, Charlton is of the opinion that phagocytic leucocytes are responsible for the ultimate destruction of the degenerating mouse egg. He describes polymorphonuclear leucocytes, varying in number, inside the zona pellucida and believes that they are responsible for the final dissolution of the ovum. An examination of a series of fixed and stained rat eggs in the present study reveals a great variability in the number of leucocytes encountered on the surface of the zona pellucida as well as within the egg itself (figs. 3 to 6). Although the number of leucocytes present was large in some instances (figs. 3 and 4), most of them were merely adherent to the surface of the zona pellucida while only a few were found within the vitellus. In others the total number of leucocytes was small and those present were found mainly within the vitellus with a few, if any, adhering to the zonae pellucidae (figs. 5 and 6).

The present observations demonstrate that uterine ova of the rat possess a zona pellucida in disagreement with Mann's ('24) statement that unfertilized ova of the rat lose the zona pellucida when they first enter the uterus.

A summary of the number of ova recovered from seventeen females examined 8 to 12 hours after the onset of heat is presented in table 2. Eggs may be recovered at this time from the Fallopian tubes, as well as from the uterus and vagina. Those in the tubes are derived from the ovulation in progress, whereas those in the uterus and vagina are degenerating ova from the previous ovulation. The number encountered in

the uterus depends upon whether the cervix has relaxed or not. It has been observed that relaxation of the contracted cervix and the escape of uterine fluid occur a few hours before termination of heat (unpublished data).

TABLE 2

Number and location of ova recovered from animals killed 8 to 12 hours after the beginning of heat

ANIMAL NO.	NUMBER OF OVA RECOVERED FROM:			
	Fallopian tube	Uterus	Vagina	Total
4	8	8	0	16
7	7	2	1	10
8	7	0	0	7
9	8	0	0	8
10	8	0	0	8
11	8	0	0	8
12	9	6	0	15
13	7	5	0	12
14	8	7	0	15
15	10	0	0	10
16	8	0	0	8
17	8	0	0	8
20	12	4	0	16
21	6	0	0	6
22	9	8	0	17
23	9	4	2	15
24	8	0	0	8
Total	140	44	3	187

Animal number 4 (table 2) is a typical example of a group of rats in which ovulation has taken place, but relaxation of the cervix has not yet occurred so that the degenerating eggs are still present in the uterus. In number 4 eight recent ova were clumped together in the tubal sacs of the oviducts and eight degenerating ova were present in the uterus. The vagina was dry and the uterine cornua were still maximally distended.

Animals number 7 and 23 are instances in which ovulation had occurred and cervical relaxation was just taking place.

Consequently degenerating ova were present in the uterus and vagina and newly ovulated ova were clumped in the oviducts.

The escape of the accumulated uterine fluids via the cervix and vaginal orifice occurs rapidly. Only in those cases in which washings are made at the precise time that the fluid of the uterine cornua begins to empty into the vagina can one expect to find ova in the vaginal segment.

In view of these findings it is clear that during the normal reproductive cycle in the rat the unfertilized ova from the previous ovulation do not undergo dissolution and resorption in the uterus, but are eliminated by being washed out of the vagina near the end of the succeeding heat period. Consequently, if an animal is examined at the proper time of the heat period, ova from two ovulations can be obtained.

The question may be raised as to the viability of the ova found in the uterine cornua, i. e., in case of insemination are these fragmenting eggs capable of being fertilized and undergoing development? Blandau and Jordan ('41) demonstrated by artificially inseminating the rat at varying intervals after ovulation that the extreme limit of ovum age from which any normal young may be expected is approximately 10 hours after ovulation, and that no pregnancies result from inseminations made 12 hours or more after ovulation. Since these uterine ova have been ovulated for approximately 72 to 96 hours, there is no possibility that fertilization could be affected.

Table 3 is a summary of the number of degenerating ova from twelve pseudopregnant females examined 96 to 120 hours after the onset of heat. The degenerating ova were consistently recovered from the uterine cornua. In another eleven pseudopregnant rats examined 120 and 144 hours after the onset of heat, degenerating uterine eggs were found in only three individuals. No ova at all were recovered from still another ten pseudopregnant animals examined more than 144 hours after the onset of heat. A striking difference in the condition of ova examined between 96 and 144 hours when compared with eggs examined earlier was that the zonae pel-

lucidae were becoming less distinct and were assuming a sticky consistency. The latter quality appeared to be responsible for large amounts of extraneous matter which became adherent to their surfaces.

It has not been possible to determine with finality the ultimate fate of the fragmenting ova in the pseudopregnant animal. If the uterine washings of these animals examined between 120 to 144 hours after the onset of heat it was often possible to detect small spherical structures varying in size, which appeared similar to the vesicles noted within intact degenerating ova. Whether these structures were the remains of the fragmenting ova could not be determined with certainty.

TABLE 3

Number of degenerating uterine ova recovered from pseudopregnant animals killed 96 to 120 hours after the beginning of heat

NUMBER OF EGGS	NUMBER OF ANIMALS
2	1
3	1
5	2
6	2
8	2
9	2
10	2
—	—
Total 43	12

The work of Hall ('36) suggests that the course of ovum degeneration may be different in the pseudopregnant animal from that encountered in the normal estrous cycle. He found that the uterine fluids are distinctly alkaline during the normal estrous cycle. The period of deciduomata development, however, was characterized by a shift from an alkaline to an acid reaction of the uterine fluid. Moreover, according to Hall ('35), if rat eggs are placed in an acidified environment, *in vitro*, within the range measured in the deciduomata, the zona pellucida became sticky and flaccid and eventually dissolved.

On the other hand, it is not inconceivable that eggs or fragments of eggs may be washed out through the vagina in a manner somewhat similar to that described for the normal cycle. This possibility is strengthened by Astwood's findings that there is a gradual increase in the water content in the uteri of pseudopregnant animals, which reaches its maximum on the fifth day of pseudopregnancy. Even though the quantity of fluid within the uterine lumen appears to be relatively small at this time it, nevertheless, may be sufficient to cause the fragmenting eggs to be washed through the vagina.

SUMMARY

During the normal reproductive cycle in the rat unfertilized ova remain within the uterine cornua until the end of the succeeding heat period. The unfertilized ova do not undergo dissolution and are not reabsorbed but are eliminated by being washed out of the vagina near the end of heat. If the reproductive tract of the female rat is examined at the proper interval, ova from two ovulations may be recovered at the same time. Recently ovulated ova will be confined to the proximal end of the oviduct, while fragmenting ova from the previous ovulation will be found in either the uterine cornua or the vagina.

In the pseudopregnant female, fragmenting ova with zonae intact were recovered from the uterine cornua for as long as 6 days after ovulation. No intact ova were recovered from animals examined after this time. It is suggested that in the pseudopregnant animal the loss of the zona pellucida is due to an increased uterine acidity which occurs at about the time deciduomata are formed.

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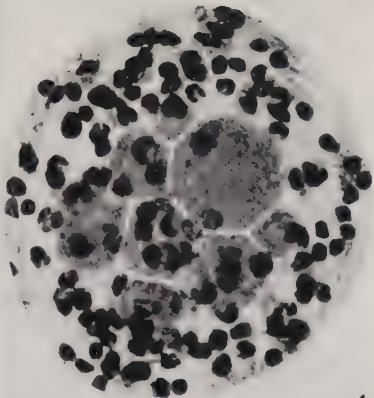
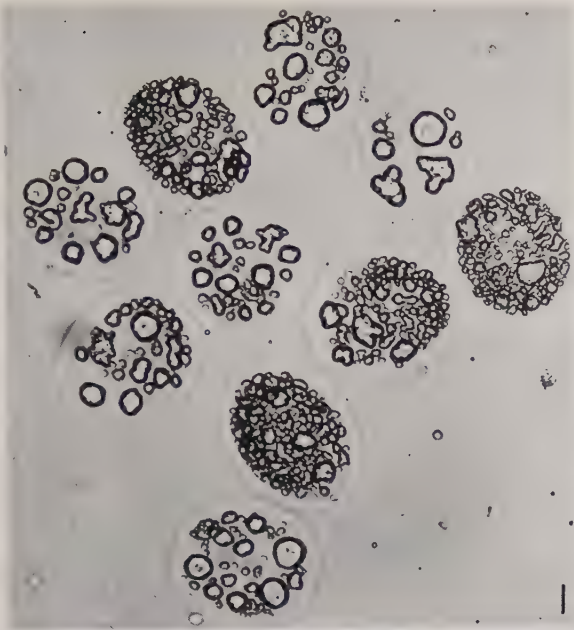
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PLATE 1

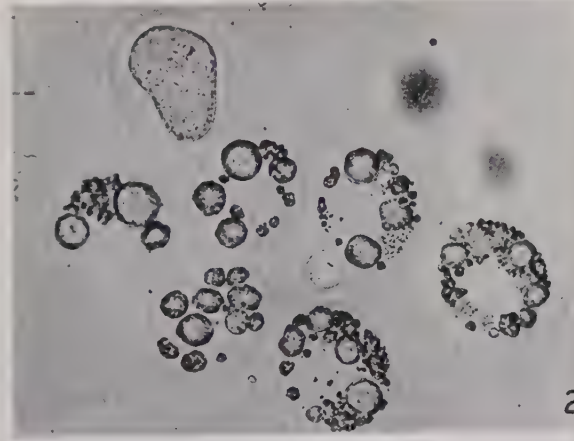
EXPLANATION OF FIGURES

1 and 2 Ova recovered from the uterine fluids of individual animals 1 to 4 hours after the beginning of heat. $\times 150$.

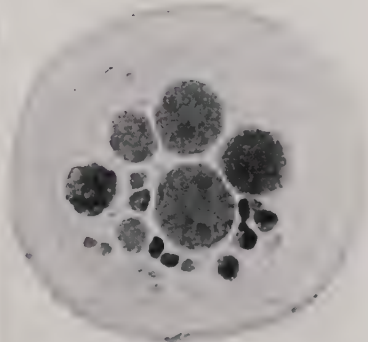
3 to 6 Whole mount preparations of ova stained with a nuclear stain to demonstrate phagocytic leucocytes. Eggs removed from the uterine cornua of animals in heat. Figure 3, $\times 150$; figures 4 to 6, $\times 400$.



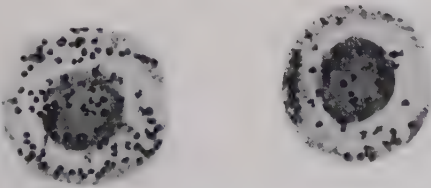
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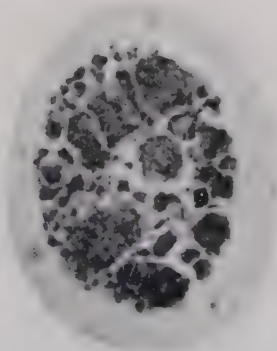
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6

SOME GROSS STRUCTURAL AND QUANTITATIVE ASPECTS OF THE DEVELOPMENTAL ANATOMY OF THE HUMAN EMBRYONIC, FETAL AND CIRCUMNATAL SKELETON ¹

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TWO PLATES (TEN FIGURES)

INTRODUCTION

Researches of the macroscopic human prenatal and circumnatal osseous skeleton are generally concerned with (1) the determination of the normal number and the time of appearance of the ossification centers and their variations, (2) the use of the skeleton as an index for the determination of age, and (3) the developmental anatomy of one or more individual bones. The few published studies interpreting, by direct observation, these phases of skeletal growth have been made on specimens prepared by dissection.³ In none has the staining and clearing method been used in studying the "in situ" skeleton. Few have presented any general principles of the developmental anatomy of the prenatal and circumnatal human osseous skeleton.

With this in mind, the present work was undertaken, to analyse this "in situ" development by direct observation, to correlate these observations with the literature of the subject,

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² Now at the University of Georgia, School of Medicine, Augusta, Georgia.

³ The roentgenographic studies are indirect observations.

and to find some general principles of the developmental anatomy of the osseous skeleton during these periods. More specific aspects of this problem will be presented later.

On the basis of the techniques used to investigate the developmental anatomy of the prenatal and circumnatal human osseous skeleton, the history of the literature may be divided into two periods; that before 1895, and that since 1895.

The literature before 1895 was based primarily on observations of gross dissected embryos, fetus and newborn infants. Kerkring (1670, 1717), Albinus (1737), and Rambaud et Renault (1864), the outstanding workers of this period, described the prenatal skeleton in much detail, both verbally and pictorially. Their numerous illustrations are in many respects far superior to any other skeletal figures ever published.

The literature after 1895 is based on researches using one of three techniques: The roentgenographic method, which has been extensively employed; the clearing-staining methods which have been used to a much less extent; and the sectioning and reconstruction technique, which has hardly been applied at all.

Dorland and Hubeny ('26), Ruckenstein ('31) and Koff ('32) present summaries of the roentgenographic literature and its relation to this problem. The use of the clearing and staining methods is found in Mall ('06) on the skeleton up to 100 fetal days; Horand ('08) on the bones of the extremities; Augier ('13, '31 and later) on the skull bones; Vallois and Cardenat ('26) on the premaxillary and maxillary bones; Wissmer ('27) on the mandible; La Coste ('31) on the occipital bone; Inman ('33) on the skull bones; and Inman and Saunders ('37) on the frontal bone. The sectioning and reconstruction technique has been utilized by Macklin ('21) to interpret the osteology of the 43-mm. CR skull, by Low ('09) for the development of the mandible, and by Hanson ('20) for the early ossification of the clavicle.

The determination of the number of ossification centers that form certain bones, the determination of the time of the appearance of these centers, the developmental anatomy of the

individual bones, and the use of the skeleton to determine fetal age form the subjects of practically all papers in the literature of this field. The technique used, the age of the specimens examined, the use of postnatal anomalies to interpret prenatal normalities, and the inaccurate determination of the age of the fetus examined are the basis of most of the controversies in the literature.

Weber (1830), Rambaud et Renault (1864), Bardeen ('10), Bryce ('15), Sammon ('23), Ruckensteiner ('31), Koff ('31), De Beer ('37) and Krogman ('41) and the standard bibliographical references are the major bibliographical sources for the study.

MATERIAL AND METHODS

The series of 40 specimens from the 25-mm. CR stage to birth was distributed as follows: 2 in the second lunar month; 12 in the third; 8 in the fourth; 5 in the fifth; 4 in the sixth; 4 in the seventh; 1 in the eighth; 2 in the ninth; 1 in the tenth lunar month; and 1 at term. Ten late fetal and newborn infant skulls, the ages determined from the formulae computed by Scammon ('37), were dissected and examined.

The specimens were stained and cleared by the following technique:

1. Fetus larger than 50 mm. CR are skinned, eviscerated, decerebrated and defatted by dissection.
2. Fix specimens in 95% alcohol from 2 weeks to 3 months, depending upon the size of the specimen.
3. Bleach in a solution of 9 parts of 95% alcohol to 1 part of hydrogen peroxide.
4. Return to 95% alcohol until ready for clearing.
5. Clear and stain simultaneously in KOH solution plus alizarin red S (alizarin red S is added to the KOH solution until the liquid is a pale lavender color).
 - a. Two per cent KOH in distilled water for small specimens (up to 150 mm. CR).
 - b. Five to 10% KOH in distilled water for larger specimens (160 mm. CR up to newborn infant size).

The clearing may take from 3 to 4 days for small specimens to 2 to 3 weeks for newborn infants. Solutions are changed every day or so and more stain is added as it is removed from solution by the bones. If the stain is applied in weak concentration, as here described, there is never any staining of the tissues surrounding the bone and destaining is never necessary.

6. When the tissues have reached the proper degree of maceration, are completely cleared and the bones are clearly visible, the skeleton is thoroughly stained. Specimens may then be dehydrated by the addition of increasing concentrations of glycerin. Specimens are stored in pure glycerin.

As alizarin red S is a stain that is specific for calcium salts (Cameron, '30) and as calcium salt impregnated osseoid tissue is bone, it may be concluded that in specimens properly cleared and stained all prenatal and circumnatal bones will be stained red.

OBSERVATIONS AND DISCUSSION

1. Definitions (plate 1)

Primary trabeculae ossei are the meridionally oriented long osseous spicules extending from the corpus of the bone, peripherally, into the unossified connective tissue. Secondary trabeculae ossei are the short osseous spicules which interconnect the primary trabeculae ossei. The open reticular zone is the meshwork of primary and secondary trabeculae ossei. Free bony islands are the small open reticular bony networks composed of short trabeculae ossei. These structures may attain a size of 1 mm. by 5 mm. Free bony nodules are the small dot like masses of bone. These two latter structures are detached from the bone proper. The marginal zone, just peripheral to the open reticular zone, is composed of the free bony nodules and islands. The primary central plate of a developing flat bone is the densely trabecularized thickened portion of the bone which appears as solid bone. The secondary central plate, found in some developing membrane bone (all open reticular and some smooth bordered plate bones) is the weakly trabecularized, thin portion of the bone that is

composed of a closely knit meshwork of trabeculae ossei surrounding small unossified areas (in contrast to the elongate unossified areas of the open reticular zone). In open reticular bones, this plate is located between the primary central plate and the open reticular zone. In smooth bordered plate bones, this secondary central plate, if present, is eccentrically located and surrounded completely by primary central plate. In the long bones, the shaft is morphologically equivalent to the central plate.

Each prenatal bone is stained differentially by the alizarin. The intensely stained regions are the alizarin trabeculae ossei, the terminal disks of the epiphyseal end of the diaphyses of the tubular bones, certain portions of the peripheral margins of the flat, irregular cartilage bones, groups of trabeculae called fascicles, and certain thickened regions of certain bones. All of these structures probably represent the sites of recent calcification, as alizarin "reacts readily in vitro and in vivo with recently deposited calcium phosphate or carbonate, normal or pathological, but often fails to stain older deposits." (Cameron, '30.)

"Alizarin" trabeculae⁴ are the alizarinophilic trabeculae ossei and the alizarinophilic trabecular appearing structures on the superficial surfaces of the central plate and shafts. For the purposes of this discussion trabeculae are only those structures, whether true bony trabeculae or not, that by virtue of the selective alizarin affinity by the bone, form trabecular appearing patterns which stain intensely red. The alizarinophilic trabeculae ossei include the primary and secondary trabeculae ossei and the free bony nodules and islands. In each bone the trabeculae form definite primary and secondary patterns. In the open reticular bones the fine network of the trabeculae of the superficial surface of the central plate is continuous peripherally with the trabeculae of the reticular zone. The trabeculae of the central plate of the smooth bordered plate bones are continuous with the trabeculae ossei of

⁴ Unless otherwise specified, hereafter "trabeculae" mean "alizarin" trabeculae.

the open reticular zone, when the latter is present. The trabeculae of the peripheral band plate bones extend into the peripheral bands, while the trabeculae of the shafts of the tubular bones extend into the terminal disks.

2. Four morphological types of developing bones

In the prenatal skeleton cleared and stained by the technique previously described, four morphological types of bones are suggested.

1. *Open reticular bordered plate bone (fig. 1).* The open reticular bones, which include the membranous calvarial bones (frontal, parietal, squamous portion of the squamosal, interparietal bones and wing of the alisphenoid, nasal, lacrimal, vomer bones and, when membranous, Kerkring's ossicle), are composed of primary and secondary central plates bordered by a well developed open reticular zone and, in most cases, by a marginal zone.

The development of an open reticular bone proceeds in a definite sequence. Soon after the appearance of its ossification center the bone has a reticular structure of a fine meshwork of primary and secondary trabeculae⁵ in the form of an open reticulum. A marginal zone of free bony nodules and free bony islands, variable in their size, quantity and location, is found in most open reticular bones soon after the appearance of the center of ossification (figs. 5 and 6, parietal bone). By the third lunar month (fig. 7, parietal bone) the oldest portions of each open reticular bone (in the region of the site of each ossification center) loses its open reticular structure as the unossified spaces of the reticulum are ossified to form primary and secondary central plates containing no diplöe. Peripheral to the central plate is the open reticular zone and peripheral to that, usually, the marginal zone. Thus commencing at the site of original ossification and proceeding peripherally, the bone is progressively less densely ossified. Free bony nodules and free bony islands are associated mainly with the frontal, pari-

⁵ Not "alizarin" trabeculae.

etal and interparietal bones along the metopic, coronal and lambdoidal sutures, and with the parietal bone along the squamosal suture. The other open reticular bones rarely have bony islands but they often possess free bony nodules. By the fourth lunar month (fig. 9) the central plate region has enlarged both relatively and absolutely as compared to the other zones, and the open reticular zone is a narrow peripheral band. The marginal zone, if present, is generally restricted to a few bony nodules, and free bony islands are usually absent. At birth (fig. 10) the thickened central plate region forms practically the entire bone. Surrounding the plates are the short peripherally directed ossified spicules of the narrow open reticular zone. A few free bony nodules and an occasional sutural bone (which may represent a modified free bony island) represent the marginal zone.

During prenatal life an open reticular bone has three structural zones: (1) The central diplöeless plate of bone which is the body of the bone, (2) the open reticular zone which is peripheral to the central plate, and (3) the marginal zone of free bony nodules and islands which is peripheral to the open reticulum. The central plate (including primary and secondary plates) increases in both absolute and relative area with respect to the open reticulum and marginal zones. The free bony nodules and islands vary in size from small masses approximately 0.5 mm. in diameter to large ones 1 to 5 mm. in diameter. As fetal age progresses these nodules and islands are incorporated into the bone. The nodules are irregularly distributed on all peripheral borders, but the islands are restricted primarily to the borders, of the coronal, lambdoidal and sagittal sutures. With age the central plate enlarges and the peripheral border and marginal border are reduced in width.

The trabeculae of the frontal bone radiate from a center in the middle of the frontal squama, into the squama; and from a line at the middle third of the supraorbital crest posteriorly into the orbital facies. The center of radiation of the frontal squama rises with age. The trabeculae of the parietal bone radiate from the morphological center of the bone. In each

parietal bone ossifying from two centers, the two centers of radiation persist until the two bony entities fuse into one. In the latter stage the parietal bone has one center of radiation obscured by the reticular network of the trabeculae in the center of the bone. From the central portion of the base of the temporal squama, the trabeculae radiate superiorly, anteriorly and posteriorly into the squama. The vomerine trabeculae radiate from a line in the middle third of the junction of the squama and keel anteriorly, superiorly and posteriorly into the squama; and anteriorly, inferiorly and posteriorly into the keel. The trabeculae of the lacrimal and the nasal bone generally form a reticular network. The interparietal trabeculae radiate superiorly and laterally from the central third of the bone's inferior border into the squama. In Kerkring's ossicle the trabeculae extend inferiorly from the superior apex to the base of the bone. The trabeculae of the membranous part of the alisphenoid bone radiate distally from the region just anterior to the incisura of the foramen ovale and foramen spinosum, into the wing.

2. *Smooth bordered plate bone (fig. 2).* These complex trabecular bones, which include the maxilla, the mandible, the zygomatic bone, the palate bone, the inferior border and zygomatic process of the squamosal bone and the tympanic annulus, have two or more trabecular patterns. They possess relatively smooth borders with few, if any, bony nodules and no bony islands; and no long primary trabeculae extending into the surrounding connective tissue. Occasionally a secondary central plate is present. Growth in area is a function of the central plate margins except in those restricted regions of the short primary trabeculae ossei.

The trabecular pattern of the maxilla and mandible is complex. The trabeculae of the external facies of the maxilla radiate (1) from a center in the cuspid region, extending superiorly on the frontal process, postero-laterally toward the infraorbital crest and the postero-lateral facies of the zygomatic process, (2) from a line of the alveolar premolar region, extending superiorly on the zygomatic process and

posteriorly on the inferior border of the zygomatic arch and posterior portion of the corpus, and (3) from a center on the medial alveolar border of the premaxilla, extending to the maxillary corpus and to the inferior border of the piriform aperture. Except for the zygoma and its radiations, the trabecular pattern of the internal facies is similar to that of the external facies. The orbital facies trabeculae radiate from the anterior half of the groove of the future infraorbital canal. They extend postero-laterally into the horizontal facies, and supero-medially into the vertical facies. The palatine process trabeculae radiate from a center in the cuspid region and extend anteriorly, medially and posteriorly into the process.

From the apex of the angle formed by the symphyso-condyloid fascicle and the marginal fascicle of the mandible (Wissmer, '27) trabeculae extend posteriorly. On the inferior margin of the mandible antero-posterior oriented trabeculae extend from the symphysis to the angle. Halfway between the alveolar border and the inferior margin in the cuspid region is a center of radiation from which trabeculae radiate to the symphyseal margin. From a line of radiation extending from the junction of the alveolar border and the coronoid process inferior to the symphyso-condyloid fascicle, the trabeculae radiate proximally on the ramus to the incisura mandibulae. The trabeculae of the splenius are oriented as practically parallel trabeculae approximately parallel to the shallow arc formed by the line of the mandibular foramen, the mylohyoid line and the continuation of the mylohyoid line as an arc to the antero-superior end of the symphysis. Exclusive of the splenius the trabecular pattern of the medial facies is the same as that of the lateral facies. Wissmer ('27) has a comprehensive account of the mandibular fascicles.

From a short line of radiation in the middle of a ridge formed at the junction of the orbital and temporal facies the trabeculae of the zygomatic bone radiate anteriorly into the orbital process and postero-superiorly into the frontosphenoidal process. An infero-anterior radiation extends from the entire ridge into the maxillary process. The small areas be-

tween the frontosphenoidal and maxillary processes and the posteroinferior angle of the maxillary process are characterized by the absence of trabeculae. From a center of radiation just anterior to the bases of the pyramidal process at the angle between the horizontal and perpendicular plates of the bone, the palatine bone trabeculae radiate into the two plates and the pyramidal process. The trabeculae of the tympanic annulus and the zygomatic process of the squamosal bone spiral around the long axis of each bone.

3. *Peripheral band bone (fig. 3).* Peripheral band bones, which include the scapula, ilium, neural arches, supraoccipital, exoccipital, basioccipital, orbitosphenoid, basisphenoid, presphenoid, ethmoid, sternal centers, ischium, pubis, calcaneous, talus and other tarsal and carpal bones, are the flat and irregular cartilage bones whose peripheries have, in whole or in part, a strong affinity for alizarin red S. The densely stained band-like peripheries, sharply demarcated from the lightly stained body of each bone, have two zones: The major zone of the band, approximating the body of each bone, has a homogeneous intense red color; while the minor zone of the band is a narrow peripheral lightly staining margin of short fine trabeculae perpendicular to the long axis of the zone. These fine trabeculae probably represent both calcified cartilage and bone. The body of each bone has a simple trabecular pattern.

The trabeculae of the orbitosphenoid bone are oriented as a series of curved lines, parallel to each other and to the median borders of the bone. The peripheral band is generally present on all except the median borders. The basisphenoid, presphenoid, lingula and cartilaginous portion of the alisphenoid bones have networks of trabeculae. The peripheral bands of these bones are inconstant except for those at the anterior and posterior borders of the basisphenoid and presphenoid bone. The bands of these two bones resemble the disks of tubular bones.

From a center of radiation at the junction of the external occipital crest and the inferior nuchal line (middle of the superior border of the bone), the trabeculae of the supra-

occipital radiate inferiorly and laterally, and the inferior and lateral peripheries of the bone have a peripheral band. The trabecular pattern of the basioccipital and exoccipital bones is a network. Soon after its appearance, the trabeculae of the basioccipital bone radiate from the posterior portion of the middle of the bone extending anteriorly and laterally; the exoccipital trabeculae extend from the jugular process and the exoccipital condyle into the body of the bone. Each border of the basioccipital bone except the posterior has a peripheral band. The anterior borders of the jugular process and the exoccipital condyle and the posterior border of the body of the exoccipital bone have peripheral bands. The petrosal bone has a dense network of fine trabeculae and inconstant peripheral bands generally present in the region of the fossa subarcuata and the hiatus canalis facialis.

The trabeculae of the scapula radiate from a center located at the junction of the medial end of the spine and the body of the bone, and they extend into all regions of the bone. The vertebral, glenoid and medial acromial scapular borders possess peripheral bands (fig. 3). Each component of the sternum has a centrally located center from which the trabeculae radiate peripherally (Borovansky, '31). Each neural arch is characterized by the presence of two sets of parallel trabeculae perpendicular to each other. One row extends supero-inferiorly from the superior to the inferior articular processes, while the other row extends from the distal end of the lamina to the medial end of the pedicle, being interrupted in the middle by the first row. The distal ends of the lamina, pedicle and articular processes have peripheral bands. From a center just anterior to the angle of the greater sciatic notch the trabeculae fan out into the corpus of the ilium. All borders, except the border of the greater sciatic notch, have peripheral bands. The trabeculae of the pupis and the ischium form networks and the ends of the bones may have peripheral bands resembling terminal disks.

4. *Tubular bones (fig. 4).* Tubular bones include all the long bones of the body. They have cartilaginous epiphyses

and stain differentially with alizarin red S. The body of the osseous shaft is stained rather homogeneously, but on the surface of the shaft there is a network of subperiosteal trabeculae parallel with the long axis of the shaft. Each end of the shaft is capped by two deeply stained terminal disks. The disk closest to the body of the shaft (major zone) is stained densely and rather homogeneously by alizarin. The narrow disk of the diaphyseal-epiphyseal junction (minor zone) is composed of short fine trabeculae perpendicular to the plane of the disk. It probably represents both calcified cartilage and bone.

Conclusions. The open reticular zones, the marginal zones, the peripheral bands, and the terminal disks are the locations of the sites of proliferative growth. The central plates and the shafts are the regions of bony reconstruction and slow osseous growth. The "alizarin" trabeculae are probably manifest of the sites of recent ossification.

Although one center of ossification usually gives rise to one morphological type of bone, an ossification center may give rise to more than one (morphological) type. For example, the wing of the alisphenoid bone is an open reticular bordered plate bone while the base is a peripheral band bone.

A definitive bone may be formed of two or more of these morphological types of bone. For example, in the definitive occipital bone, the interparietal bone and Kerkring's ossicle, when membranous, are open reticular bordered plate bones; while the basioccipital, the exoccipital and the supraoccipital bones, and Kerkring's ossicle, when cartilaginous, are peripheral band plate bones.

The open reticular bordered plate bones and the smooth bordered plate bones are membrane bones, while the peripheral band bones and the tubular bones, except possibly the clavicle (Hanson, '20) are cartilage bones. There is an intimate relation between the developmental morphology and the tissue origin of the bones appearing prenatally.

Each bone stained by this technique has superficial subperiosteal lines which are stained more deeply than the matrix of the bone. These lines generally radiate from one or more

centers in each bone, or in a few cases, the lines are parallel and do not radiate from a center. A bone with one or two centers of radiation has a simple trabecular pattern. A bone that has more than two centers of radiation is considered as having a compound trabecular pattern. Each bone of the fetal skeleton has a definite trabecular pattern of subperiosteal trabeculae.

3. *Periods of development*

Three periods of development in the morphogenesis of the bones of the embryonic, fetal and circumnatal osseous skeleton are suggested. They are:

1. *The period of differentiation.* This is the time of the appearance of the ossification centers. This time of appearance, subject to variation which during the fetal period is measured in days (Pryor, '28; Koff, '32), is influenced by such factors as sex (Pryor '28; Sawtell '29; Christie et al. '41), heritable tendencies (Pryor, '39), nutrition (Francis, '40), race (Christie et al. '41), pathology (Francis, '39; Gross, '40), and parity (Adair and Scammon, '21; Christie et al. '41). Groups of ossification centers exhibiting relative independence in their time of appearance, may exist in the extremities (Robinow, '42).

From a survey of the literature, partially confirmed by original observations, the general order of appearance of the ossification centers by region is as follows: (a) diaphyses of the two proximal segments of the extremities, (b) bones of the facial region, (c) and (d) calvarial bones and the thoracic bones (ribs, clavicle, scapula and primary vertebral bones), (e) free vertebral column primary bones, (f) the diaphyseal bones of the hand, (g) and (h) basicranial bones and pelvic bones (with the exception of the ilium that appears early), (i) diaphyseal bones of the foot, (j) sternal bones, and (k) hyoid bones.

2. *The period of proliferation.* This is the time of the rapid extension of ossification into unossified regions. During this period the linear growth of a bone proceeds at a higher rate of increment than that of the corresponding body segment,

and the morphological features characteristic of each bone appear.

For the facial bones, this period extends from the period of differentiation to the 70-mm. to 80-mm. CR stage (figs. 5 to 7). Early in the period each facial bone (except the nasal, vomer, and lacrimal bones) assumes the characteristics of the typical smooth bordered plate bone. Their period of ontogeny from the trabecular center of ossification is short as contrasted to the period of ontogeny of the open reticular bordered plate bones from the reticular network to the formation of an extensive central plate late in the period of proliferation. The widths of the broad suture spaces between the facial bones are continually being reduced.

For the membranous calvarial bones, this period ends at about 140-mm. to 160 mm. CR stage (fig. 8). During this interval these rapidly expanding bones grow peripherally by the open reticular zone and the marginal zone. Inman ('33), who analysed quantitatively the growth of the membrane bones during fetal life, states that these bones grow with constant increments until the 140-mm. to 160-mm. CR stage, and subsequently at a constant increment of less magnitude.⁶ At the 140-mm. to 160 mm. CR stage, they have assumed a form resembling their definitive form and an area roughly proportional to their definitive area. The reticular zone and the

⁶ During the 140-mm. to 160-mm. CR stage to birth period (period of construction) the dimensions of the calvarial bones measured by Inman ('33) are identical to some of the external dimensions measured by Scammon and Calkins ('29). The difference between the dimensions measured by these workers during the period before the 140-mm. to 160-mm. CR stage (period of proliferation) is that Inman did not measure the unossified regions between the bones and the soft tissue over them while Scammon and Calkins did.

Whether the growth curves of the bones with respect to the external body dimensions are interpreted as rectilinear curves or as power curves does not effect the concept of the developmental periods. During the period of proliferation the curve of the various external dimensions of the body to body length and the curve of the dimensions of the bones to body length converge toward each other until the end of the period. During the period of construction these two sets of curves are roughly parallel to each other. At the stage between the two periods these two sets of curves, if straight lines, will intersect to form an angle or, these curves, if curved lines, will exhibit their greatest curvature.

marginal zone are narrow; the dominance of the central plate over the two peripheral zones is definite.

The principles of the prenatal growth of the cartilage bones including both the peripheral band plate bones and the tubular bones are summarized by Inman ('33). "The ossification centers appearing in the primordial chordocranium grow with constant increments in relation to sitting height. Throughout the body, ossification centers arising in cartilage appear to grow by constant increments; this occurs in the ossification centers for the femur, humerus, tibia, radius, ulna, metacarpals, metatarsals and the bodies of the vertebrae," . . . "The rates of growth of endochondral bones are also the rates of proliferation or activity of the epiphyseal ends of the bones (if one accepts the no interstitial bone growth hypothesis). Each group of cartilage cells contributing to the formation of a particular bone has a constant rate of proliferation when compared not with time but to the size of the fetus." Hesdoffer and Scammon ('28) and Robb and Clarke ('35) demonstrate the rectilinear relation of the growth of the femur, humerus, radius, ulna, fibula and tibia to other linear dimensions during prenatal life. Objective data of the quantitative growth of the other postcranial cartilage bones during prenatal life have not, to this author's knowledge, been presented. However, if Inman's ('33) suggestion of the constant rate of proliferation of the cartilage cells when compared to the size of the fetus is accepted for all cartilage bones during the fetal period, these bones may be classified as growing in a rectilinear relation to the body length during fetal life. The cartilage bones, except the ribs and the clavicle, grow by constant increments and do not reach definitive proportions in relation to their body segments before birth. This is qualitatively visible. Brodie's ('41) data suggests that the interval between the two periods for the basi-cranial bones may occur at $1\frac{1}{2}$ years of age. However, the power curves of the postnatal growth of the bones and the body segments may obscure the determination of the stage between these two periods.

A comparison of the cartilaginous supraoccipital bone and the membranous interparietal bone in the figures in plate 2 gives a graphic illustration of the relatively rapid growth of the calvarial bones as compared to the relatively slow growth of the cartilage bones.

3. *The period of construction.* This is the time of the osseous reorganization of each bone, and of linear osseous growth at a rate of increment approximately equal to that of the body segment with which it is associated. As previously noted, the period of proliferation ends and the period of construction begins approximately at 70 mm. to 80 mm. CR in the facial bones, clavicle and ribs (figs. 7 to 10 incl.); approximately at 140 mm. to 160 mm. CR in the membranous calvarial bones; and after birth in the cartilage bones. It is characterized by an abrupt decrease in the growth increment of the bones, with a concomitant formation of a bony border about the peripheral edges of the bone (Inman, '33); and the assumption, by the bones, of an area or length proportional to their definitive area or length (figs. 9 and 10).

SUMMARY

1. On the basis of the techniques used to investigate the development of the embryonic, fetal and circumnatal skeleton, the history of the literature may be divided into two periods: (a) before 1895, and (b) after 1895.

2. By clearing in KOH and staining with alizarin red S simultaneously, overmaceration and overstaining of human embryos and fetus can be prevented more readily than in previously published techniques.

3. Structures associated with the developing bones are defined. These include the definitions of primary and secondary trabeculae ossei, open reticular zone, free bony islands, free bony islands, free bony nodules, marginal zone, primary and secondary central plates and the structures resulting from the differential staining affinity of "alizarin" to bone.

4. On the basis of the gross structure of the developing prenatal bones cleared by KOH and stained by "alizarin" four

morphological types of bones are described: (a) open reticular bordered plate bones, (b) smooth bordered plate bones, (c) peripheral band bones and (d) tubular bones. The relation of the terms noted in (3) to these four types of bones and a brief description of the alizarin trabecular pattern in the pre-natal bones are presented.

5. Three developmental periods in the morphogenesis of the bones during prenatal life are postulated: (a) the period of differentiation or the time of the appearance of the ossification centers; (b) the period of proliferation or the time of relatively rapid osseous growth (growth of each bone at a rate greater than its corresponding body segment); (c) the period of construction or the time of relatively slow osseous growth (growth of each bone at a rate approximately equal to its corresponding body segment).

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PLATE 1

EXPLANATION OF FIGURES

Diagrams illustrating the four morphological types of human prenatal bones. The degree of intensity of the black in the figures approximates the degree of intensity of the red in the prenatal bones when stained with "alizarin red S" (degree of affinity of the dye for the bone).

1. Open reticular bordered plate bone (composite figure).
2. Smooth bordered plate bone (composite figure).
3. Peripheral band bone (scapula).
4. Tubular bone.

Legend: Ali. Trab., "alizarin" trabeculae; C.P., central plate; F.B.I., free bony island; F.B.N., free bony nodule; M.Z., marginal zone; Ma. Z., major zone; Mi. Z., minor zone; P.C.P., primary central plate; P.B., peripheral band; P.T.O., primary trabeculae ossei; O.R.Z., open reticular zone; S., shaft; S.C.P., secondary central plate; S.T.O., secondary trabeculae ossei; T.D., terminal disk.

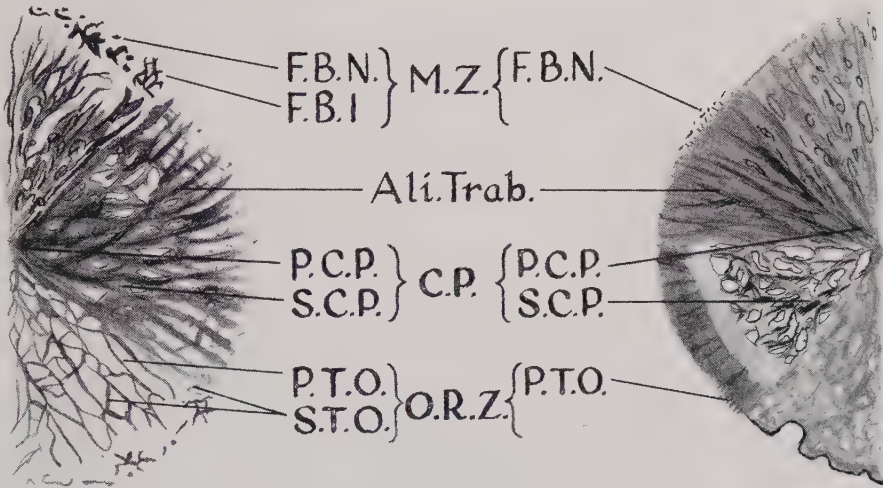


FIGURE 1

FIGURE 2

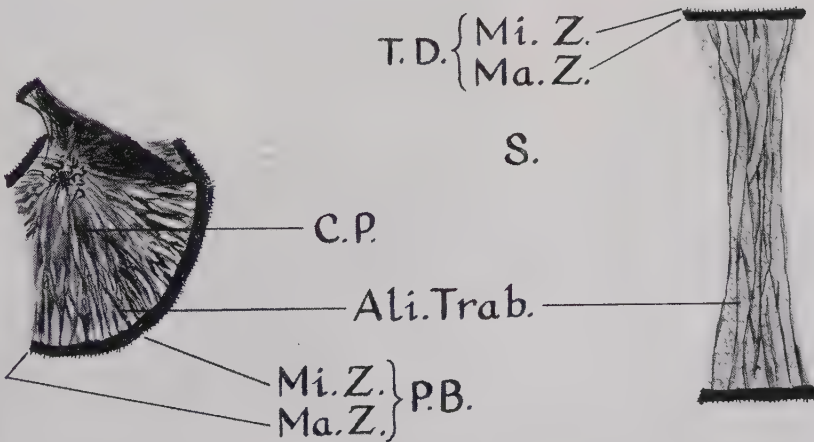


FIGURE 3

FIGURE 4

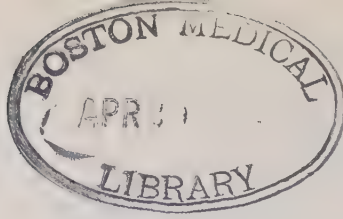


PLATE 2

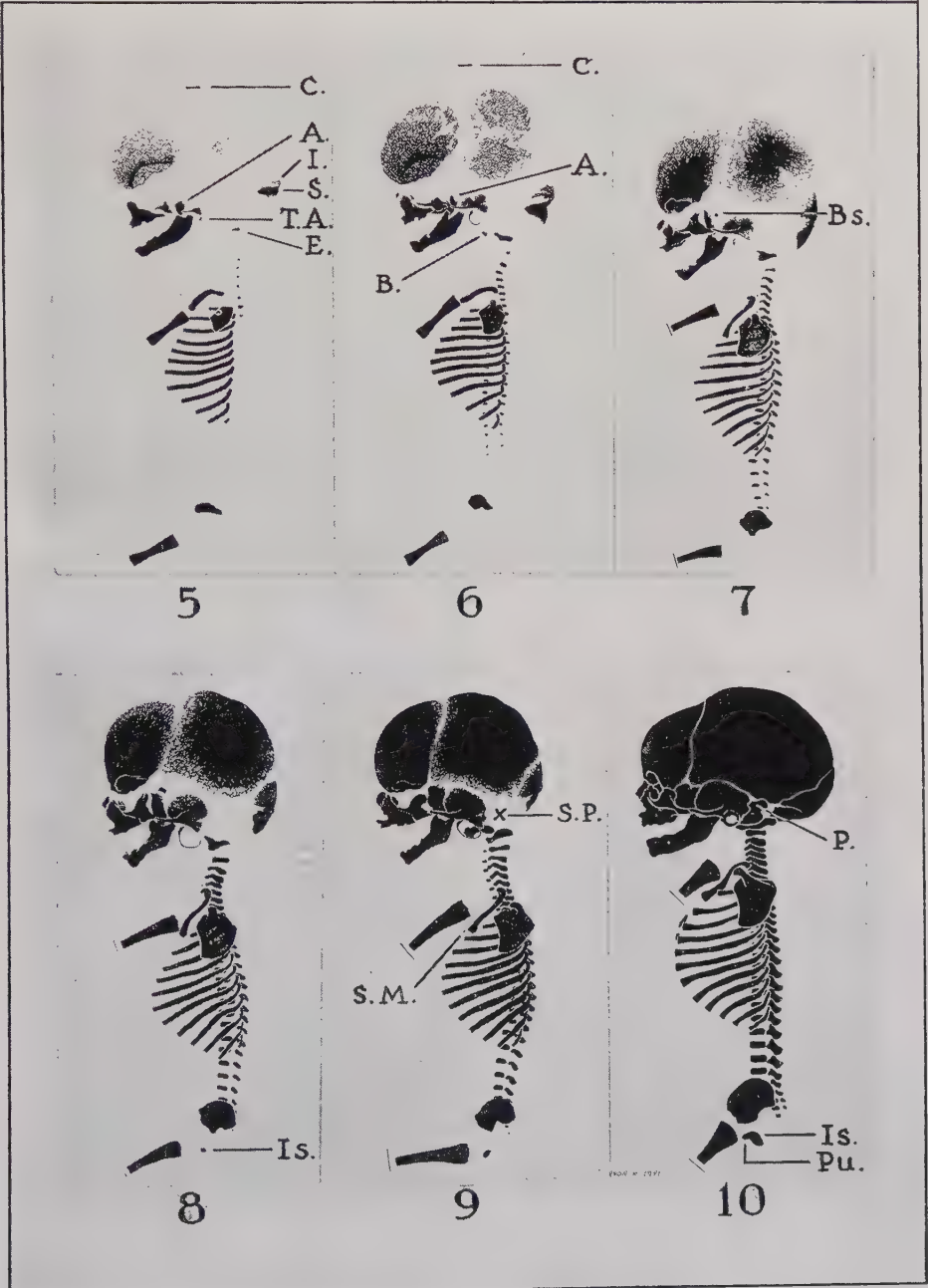
EXPLANATION OF FIGURES

Side view drawings of six stages in the development of the human prenatal and circumnatal osseous skeleton. Crown-rump lengths are held constant in all figures.

Note: (1) the maturation of the facial bones, ribs and clavicle in the stage depicted in figure 3, (2) maturation of the calvarial bones in the stage depicted by figure 5, (3) the central plate, the open reticular zone and the marginal zone of the calvarial bones, and (4) the relatively slow growth of most of the cartilage bones as exemplified by the supraoccipital bone and the relatively rapid growth of the membrane bones as exemplified by the interparietal bone.

	CR LENGTH	CH LENGTH	APPROXIMATE AGE
Figure 5	38 mm.		64 days
Figure 6	58 mm.	75 mm.	78 days
Figure 7	88 mm.	125 mm.	3.25 lunar months
Figure 8	105 mm.	156 mm.	3.5 lunar months
Figure 9	139 mm.	205 mm.	4.25 lunar months
Figure 10	330 mm.	479 mm.	10 lunar months

Legend: A., alisphenoid bone; B., basioccipital bone; Bs., basisphenoid bone; C., crown; E., exoccipital bone; I., interparietal bone; Is., ischium; P., petrosum; Pu., pubis; S., supraoccipital bone; S.M., sternal center of the manubrium; S.P., site of the pertosum; T.A., tympanic annulus.



A CASE OF CONJOINED TWINS IN THE PIG

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FIVE FIGURES

Although many studies have been conducted on the morphology and morphogenesis of conjoined twins in the various branches of the animal kingdom, every new case of incomplete twinning is of value as it may throw new light upon the general problem of teratology as well as the mechanisms of normal development. That we need more detailed descriptions of conjoined twins in mammals is clearly shown by the relatively small number of comparative studies now available in teratological literature. Because of the need for such studies and in order to facilitate the establishment of a more accurate classification of conjoined twins in general, this report is submitted.

Among the cases of conjoined twins in domestic animals that have been described in the literature since 1900, the writers have seen descriptions of 30 in pigs, 5 in calves, 4 in lambs, 4 in goats, 3 in rabbits, 1 in dogs and 6 in cats. Space does not permit reference to all these cases, but anyone interested will find most of them reviewed in articles by Baumgartner ('28), Glaser ('28) Nordby and Taylor ('28) and Engel ('31). Glaser ('28) citing Teruffi, lists 42 sheep, 38 cats, 31 pigs, 13 hares, 8 calves, 7 goats, 5 dogs and 5 rabbits as the numbers of conjoined twins described in the literature prior to 1900. There are no doubt other reports both prior to and since 1900 but descriptions of them are not accessible.¹

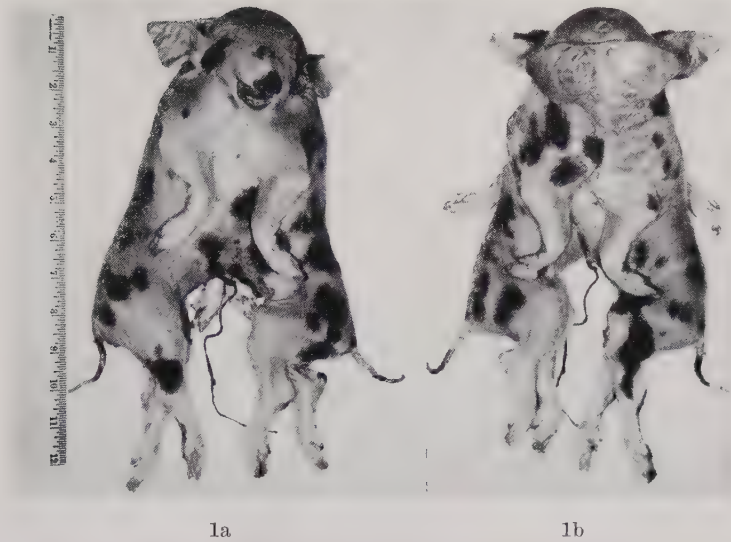
¹ A guinea pig, the only recorded among more than 100,000 born in the genetics experiments of the Bureau of Animal Industry, was, in its outward appearance, like the pig described in this report. There was no dissection made of the guinea pig. A photo of the dorsal and ventral aspects is shown in figure 2.

HISTORY OF THE PIG

The cephalothoracopagus described herein was farrowed on January 1, 1943, at the Animal Disease Station, Beltsville Research Center, Beltsville, Maryland, by a yearling sow of Large Black-Landrace breeding, produced in the herd of the Animal Husbandry Experiment Station. According to information given by Dr. L. O. Mott who kindly made the specimen available to the writers January 22, 1943, the monster was farrowed at full term (116 days) with ten pigs, five of which, including the case under consideration were stillborn. The twins were males and weighed 1075 gm. One member was somewhat smaller than the other but both were well developed as judged from the condition of their hair and development of their teeth. Unfortunately, it was not possible to make as detailed a study of the various internal structures as was desired because of the partial decomposition which set in prior to the time the specimen could be examined. Decomposition, however, was not sufficient to detract from the accuracy of the findings. For convenience, the position shown in figure 1a is referred to as the ventral aspect and that in figure 1b as the dorsal aspect.

EXTERNAL FEATURES

The twins were joined ventrally from the umbilical cord forward. Posterior to the umbilicus, the two components presented a normal appearance. There was a single head with one snout and two eyes, but with four ears, two of which were fused at the external acoustic meatus on the mid-dorsal surface near the base of the skull. Anterior to the fused ears (about 1.5 cm.) there was a proboscis-like structure which, on dissection, was determined to lead into an opening of the skull representing a rudimentary orbit. Though apparently normal in its anterior appearance, the head was broader than normal in the occipital and parietal regions. Each member had two fore legs and two hind legs in apparently normal position. All toes were normal. Each member had an anal



Figs. 1a and 1b Ventral and dorsal aspects of the cephalothoracopagus pig described in this paper.



Fig. 2 Ventral and dorsal aspects of a cephalothoracopagus guinea pig mentioned in the footnote of page 53.

opening and a penis. The left twin possessed six rudimentary teats on the right and four on the left, while the right twin had five on each side. As regards the coat color pattern, a picture of which is shown in figure 3, it is of interest to note that while both members had a uniform red coat with black spots, the number, size and distribution of the black spots

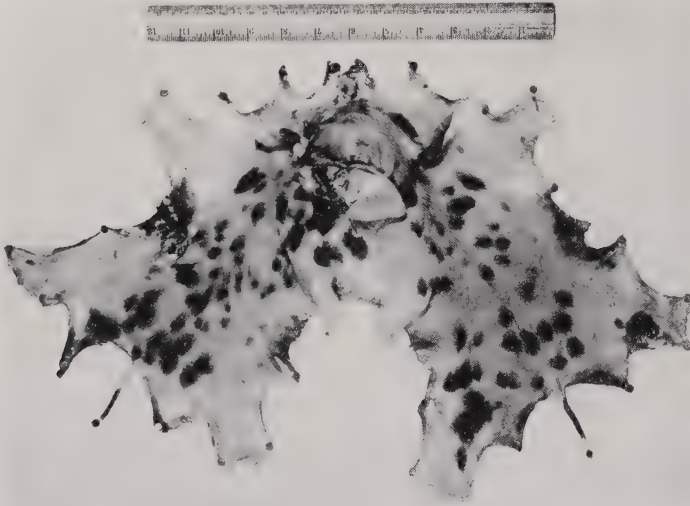


Fig. 3 Skin of the conjoined twins, showing the color pattern of the two parts.

were not identical for the two members. This apparent lack of symmetry is particularly interesting in view of the fact that the twins undoubtedly arose from a single fertilized egg and hence any variation in the two components must have been due to environmental conditions.

INTERNAL FEATURES

The muscles of the cervical and thoracic regions were the same on both sides of each twin and their origin appeared to be normal on both the ventral and dorsal aspects. Though somewhat altered in their relations to each other, all muscles on the ventral aspect were normal as regards their attachments while most of those on the dorsal aspect revealed modi-

fications in both their course and insertions. The muscles on the dorsal aspect, moreover, were somewhat longer in general than those on the ventral aspect, evidently due to the greater curvature of the neck in that region. There were two pairs of parotid glands, a large pair on the ventral surface and a rudimentary pair dorsally under the sterno-hyoideus muscles. A pair of submaxillary glands was present on the ventral surface but none were identified dorsally.

Dissection of the specimen revealed a large roomy abdominal cavity continuous anteriorly with the thoracic cavity through three separate slits in the common diaphragm, one in the left and two in the right twin. Posteriorly the abdominal cavity was continuous with the pelvic cavity of each twin. There were two livers both of which were attached to the common diaphragm and common stomach. One liver lobe protruded into the thoracic cavity of the left twin through one of the openings in the diaphragm, while the remaining lobes were in contact with the abdominal wall along the ventral aspect of the two parts. Besides containing a liver lobe, this cavity contained a part of the intestines as well as a lobe of the lung of the left twin. No abdominal organs protruded through the other two diaphragmatic slits that led into the thoracic cavity of the right twin. The stomach lying medially in the anterior part of the abdomen was somewhat larger than usual and there were two diverticuli, one on the left and one on the right cardiac side, indicating that the stomach probably arose from two stomachs fused at the median line. A single oesophagus entered the stomach on the anterior side approximately mid-way between the two diverticuli. The pylorus was at the opposite end of the stomach near the center of what probably was the line of fusion of two fundic regions. Because of tearing, the course of the intestines could not be traced throughout their whole length but there was only one small intestine immediately posterior to the stomach and two large intestines which were the result of a branching of the small intestine anterior to the entry of the ileum into the caecum. Pancreatic as well as splenic structures were identified but

the condition of the specimen did not permit the identification of more than one spleen. The genito-urinary organs were double throughout. The left kidney of the right twin was considerably smaller than the other three kidneys and only the right testicle of the left twin was in the inguinal canal just below the adominal ring. The other three testicles were still in the abdominal cavity. A urachus led from the urinary bladder of each twin to the common umbilicus. Not all vessels transverseing the umbilical cord could be determined, but since two umbilical veins were identified, it would seem that each member had its own blood supply through the common cord.

Each member had a thoracic cavity separated into two pleural cavities along the median plane by a thin membrane confluent posteriorly with the common diaphragm. Dorso-ventrally the two thoracic cavities were separated from each other by the two pericardial sacs occupying a position immediately below the sterni formed by the junction of one set of ribs from each twin. There was a lung in each of the four pleural cavities. Three of these cavities, as noted above, were continuous with the common abdominal cavity, but only the pleural cavity of the left twin facing the ventral aspect contained abdominal structures in addition to a lung. The lung in this latter cavity was about one half as large as the ones facing the dorsal aspect of the same twin and the ventral aspect of the right twin. The lung facing the dorsal aspect of the right twin was intermediate in size. Two tracheae were present. Both proceeded in the plane formed by the two sterni, but instead of leading to the two lobes of the same lung, each led to one lobe of each of the two lungs.

The specimen possessed two hearts, a large fairly normally shaped heart situated ventrad with its apex occupying a position well within the thorax of the right twin and a small flattened heart situated dorsad in a separate pericardium with its apex occupying a central position between the two thoracic cavities. Each heart had two atria and two ventricles and there was an anastomotic branch between the aortic arches of the two hearts. The aortic arch of the ventrad heart pro-

ceeded toward the left twin and that of the dorsad heart toward the right twin, while the posterior vena cava derived from the left twin proceeded to the dorsad heart and that from the right twin to the ventrad heart. Anastomosis between the two circulatory systems was thus provided by the connection between the aortic arches as well as through interchange within the body tissues. Pulmonary arteries and veins were situated as usual but their course could not be determined.

Dentition in this specimen was normal. There were two temporary tusks or "needle teeth" in each right and left maxilla and mandible. A double fissure, representing a cleft palate,

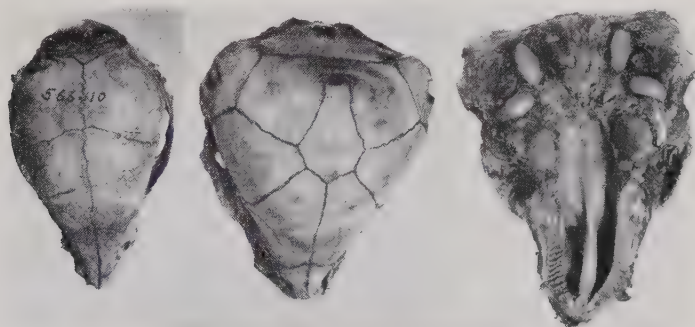


Fig. 4 Left, dorsal aspect of normal skull; center, dorsal aspect; and right, ventral aspect of the skull of the conjoined twins.

extended from the premaxillae back to the pharynx. There was complete duplicity of the larynx, one situated as usual and the other dorsad with all structures rotated at an 180° angle. A single oesophagus was situated between the ventral and dorsal larynx and proceeded back between the two tracheae. The tongue had a median furrow that was especially pronounced at the apex. In its anterior portion the tongue was wider than usual and the lateral margins were distinctly serrated.

Figure 4 shows the dorsal and ventral aspects of the skull, together with a normal skull for comparison. From the frontals forward the skull seemed normal except for a slight cur-

vature of the premaxillary and nasal portion toward the left. Posterior to the frontals were two parietals, separated from each other by an unequal-sided heptagon-shaped bone, the most posterior side of which formed the margin of a small circular opening. Dorsal to this latter bone and the two parietals was an irregularly shaped bone extending across to the occipital bones and posterior to it was another bone also extending across to the occipitals. The occipitals in general appeared normal, each possessing two condyles alongside the two foramina magna. An irregularly shaped opening was located in the center of the most posteriorly situated bone of the skull, representing the fused external auditory meatuses. The other external meatuses were located normally.

Viewing the skull from the rear it is evident that the bones of the right side belong to the right twin while those on the left side belong to the left twin. The conclusion to be drawn from the position of the three abnormally shaped bones at the back of the skull is that the most posterior one containing the fused auditory meatuses represents fused temporal bones, the one anterior to it fused parietals and the heptagonal shaped bone fused frontals. The identity of the fused temporals was further confirmed by the presence of small ossae bullae along the mid-ventral axis. From these relationships it may also be concluded that the small circular opening anterior to the fused auditory meatuses represents modified orbits.

The brain was enclosed in a single dura mater. Two olfactory lobes were present. The cerebral hemispheres were widely separated behind with two smaller fused hemispheres between them. The two cerebellar lobes were situated just back of the two hemispheres of each brain. Ventrad, the brain showed no signs of doubling. There were two pituitaries, one on each side of the mid-ventral line of the brain. There was no optic chiasma, the optic nerve to each eye leading directly from the optic tract on the same side of the brain. There was an optic nerve leading from the small orbit in the rear of the skull to the dura mater, but its course could not be traced

further into the brain. Four auditory nerves were present. Other nerves, while probably present, could not be identified.

As shown in figure 5, a vertebral column led from each occipital bone. The planes formed by the wings of the two



Fig. 5 X-ray photograph of the twins viewed from the ventral side.

atlas bones slanted ventro-laterally and the lateral cranial articular facets were larger than those medially located. On the right twin cervicals 2 and 3 were completely fused on the left, and with 4 on the right side, producing a slight rotation and a curvature of the vertebral axis to the right. In the left twin cervicals 2 and 3, and 4 and 5 were fused on the right side, while all four were fused on the left, producing a rotation and bending to the left. Cervicals 5, 6 and 7 of the right twin and cervicals 6 and 7 of the left twin appeared normal. Each twin had fifteen pairs of ribs and six lumbar vertebrae, all apparently normal.

DISCUSSION

The principal questions naturally raised by the conjoined twins are two-fold. The first question aims at determining the cause or causes underlying the twinning condition and the second question aims at establishing the period during which the twinning process began. As regards the nature of the cause or group of causes responsible for the twinning process, the possibility of course exists that it is a mutation or due to chromosome aberrations of some sort. The fact that the condition never appeared before among some 40,000 pigs farrowed at the Beltsville Research Center during the last 25 years is in harmony with this view, yet there is no evidence for such a conclusion. If the condition was caused by a retardation of the normal rate of development during a critical stage as Stockard ('21) found to obtain for the occurrence of double monsters in fishes and Newman ('23) in starfishes, any part which genetic factors did play probably was to determine differences in the susceptibility to conditions tending to retard development at a critical stage. As suggested by Stockard ('21), changes in the supply of oxygen would seem to be the most probable cause of interrupted embryonic development. In harmony with the views expressed by Wright and Eaton ('23) as regards the occurrence of otocephaly in the guinea pig, it is reasonable to assume, then, that any genetic factors which did play a part in the twinning condition prob-

ably were factors causing the developing embryo to be highly susceptible to inhibiting agents among which chance variations in implantation would appear to have been most important.

Although the true cause of their genesis remains unknown, there can be no doubt but that the conjoined twins arose within a single fertilized ovum instead of by the partial fusion of two originally separate blastocysts. That this latter possibility may safely be ruled out is shown not only by the high degree of similarity in the outward appearance and structural arrangement of the two components but is also supported by the findings of others, particularly Corner ('22) who is opposed to the view that monochorionic twin pigs may arise from the fusion of separate blastocysts.

Assuming that the deformity was due to developmental arrests as postulated by Stockard ('21), it is possible to estimate the developmental period during which the twinning process arose. Before discussing this point, it is of interest to note that not all workers are in agreement as to the mechanism by means of which incomplete twinning first becomes manifest. According to Stockard ('21) all double monsters in nature arise by a double gastrulation or blastopore formation which is initially a process of double instead of single axis formation and he is inclined to rule out the possibility of a separation of the primary blastomeres. Contrasted to this view is that of Newman ('40a) who, working with chick embryos, believes that fission followed by a secondary partial fusion of a single embryonic axis represents one of the modes of twinning. Based on movements observed along the longitudinal axis of chick embryos, Waddington ('41) on the other hand, believes that posteriorly forked twins can more readily be explained as the result of double invagination of the endoderm than as splitting of the axis, and that anterior forkings are probably due to splitting of the invaginating endoderm or of the elongating primitive streak. While the conjoined twins reported herein throw no light on this question, it is reasonable to conclude that the twinning process started at a

somewhat later stage than twinning processes resulting in completely separate twins, for according to Newman ('40b) the longer the twinning process is postponed the greater the probability that the results will be conjoined instead of completely separate twins. Since, according to Corner ('22) twinning resulting in the formation of separate pigs is already apparent before the formation of the amnion, the conclusion to be drawn from his, as well as Newman's findings, would seem to be that the incomplete twins described herein arose toward the later stages of amnion formation but before the amniotic cavity had been completely formed. This explanation seems the most reasonable one to make for it eliminates the necessity of assuming the fusion of separate amniotic cavities as would have had to occur had each component started with a separate amnion. Expressed in terms of time, twinning probably began some time between the twelfth and fourteen days after fertilization. This, according to Patten ('31), is the time when amnion formation normally occurs in the pig.

SUMMARY

A double monster pig, the first of its kind to be observed among some 40,000 pigs farrowed at the Beltsville Research Center during the last 25 years, is described with regard to its external appearance and internal structures. The specimen was of the cephalothoracopagus type. The conclusion is drawn that the twinning process first asserted itself toward the later stages of the formation of the amniotic cavity.

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THE AGE FACTOR IN THE RESPONSE OF BONE TISSUE TO ALIZARIN DYES AND THE MECHANISM OF DYE FIXATION

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INTRODUCTION

The coloring of growing bone with madder, first experimentally studied by Belchier (1736), came into general use in ossification studies after the work of Du Hamel (1739). Within the last two centuries numerous authors have studied the mechanism of dye fixation in bone first using madder (Bazano, 1738; Haller, 1747; MacDonald, 1799; Paolini, 1841; Serrès and Doyère, 1842; Brullé and Hugueny, 1845), or, later, one of its coloring principals, alizarin (Flourens, 1847; Lieberkühn, 1864), and, finally, a synthetic soluble derivative, sodium alizarin sulfonate (Gottlieb, '14; Proell, '26; Cameron, '30; Proell and Diener, '33; Schour, '41). The conclusion drawn from the early studies was that the dye becomes fixed in the calcium salts which are deposited during treatment. This hypothesis was presented for the first time by Rutherford, before 1798. Some authors, Paolini, Serrès-Doyère, and Flourens, considered the coloration due to the staining of calcium phosphate. Coloration of the inorganic substrate by madder during the calcification process was accepted by all authors, with the exception of Strelzoff (1873) and Katschenko (1882), who considered the fixation as taking place in the organic matrix of bone.

Most of the recent authors ascribe the fixation of alizarin to its affinity for "newly formed" calcium salts or "free calcium". Under this assumption, the extent of coloration served

to explain the process of calcification. According to Gottlieb ('14) the variation in coloration intensity in different parts of bone is due to progressive chemical stages of calcification. According to Macklin ('17) the areas more deeply stained are composed of freshly deposited calcium salts. According to von Möllendorf ('20) the coloration is due to the formation of calcium alizarate in bone. Proell ('26) considered alizarin as an indicator of "free" calcium in bone. Cameron ('30) suggested that "the nature of the arrangement of the calcium complexes" may be different in young and old bone. Proell and Diener ('33) affirmed that the colored zone consists of freshly deposited calcium alizarate. Schour ('41) interprets the coloration as being due to a selective staining response of the calcifying or calcio-receptive zone of the collagenous matrix.

Previous in vitro studies have shown that the fixation of alizarin, or its synthetic analogues, is a process of adsorption taking place between bone calcium phosphate and dye. The dye anion is adsorbed, while PO_4 anion passes into solution (Ercoli, '38).

In vivo the reaction is considered to take place with the calcium phosphate previously laid down and it may be assumed that the reaction will occur not only in growing but also in adult bone, irrespective of age. It was the purpose of this study to investigate the conditions of fixation of osteotropic substances with relation to the age of the animal and to analyze the mechanism of dye fixation in bone.

EXPERIMENTAL

Material and methods

Albino mice of different strains were used in the experiment. Adult animals were more than 7 months of age and weighed from 24 to 31.5 gm. These animals had not increased in weight for a period of 10 days preceding the treatment with dye. Growing animals were chosen from a group of mice 14-21 days old and weighing 8 to 11 gm.

Alizarin Red S (sodium 1.2.dihydroxyanthraquinone-3-sulfonate) was used as the osteotropic dye. The concentrations were so adjusted that the dosage could be administered in 0.3–0.4 cc. per mouse. Injections were made into the tail vein except in those groups which received subcutaneous injections (the latter are tabulated separately). The experiments on the young and adult mice were done simultaneously. Forty-eight to 72 hours after injection, when elimination of the dye was complete, the animals were sacrificed by jugular bleeding. The bones were cleaned as much as possible and placed in saline for 3–4 hours, then under running tap water for 18–20 hours. After this they were put into a 10% solution of sodium hydroxide. The adhering tissue was removed by agitating in alkaline water (pH 8–8.5). After rinsing in tap water, the bones were placed in 95% alcohol for 10 minutes and then dried. Since gross observation shows the coloration more definitely than microscopic examination of sections, the observations here reported were made with the dissecting microscope.

GROWING ANIMALS

Intravenous injection

Mice were arranged in several groups and each mouse was given one intravenous injection of Alizarin Red S. The individual doses were 20, 30, 40, 50, 60, and 80 mg./kg. respectively. The results are reported in table 1A. The following zones were found to be colored in the skull: the condyloid process of the mandible; the crest between the condyli; the zygomatic process; the alveolar part of the maxillary; the premaxillary; the laminae of the posterior part of the ethmoid bone (there was no coloration in the cribrosa); in the pelvis, the crest of the ilium, the tuberosity of the ischium, the descending ramus of the pubis; also the articular processes of the vertebrae (the bodies showed only diffuse coloration); the sternal part of the ribs; the epiphyses of long bones; the deltoid tubercle; the olecranon; the linea aspera; and the junctions of the lamellar bones.

TABLE 1

*A. Fixation of Alizarin Red S in bone of growing mice.
Intravenous injection.*

DOSE MG./KG.	NO. MICE	AVERAGE WEIGHT GM.	NO. COLORED	NO. FAINTLY COLORED	NO. UNCOLORED
(0) control	4	11	..	..	4
20	9	9.1	4	2	3
30	6	10.2	2	..	4
40	7	8.5	6	..	1
50	6	9	6	..	..
60	3	10.5	3	..	..
80	3	10	3	..	..

*B. Fixation of Alizarin Red S in bone of adult mice.
Intravenous injection.*

0	4	27	..	..	4
20	7	27	1	1	5
30	4	27	1	1	2
40	6	30	2	1	3
50	4	25	2	1	1
60	4	25	2	..	2
80	5	24	4	..	1
120	1	28.5	1		
200	2	28	2 *		

*C. Fixation of Alizarin Red S in bone of growing mice.
Subcutaneous injection.*

0	2	11	..	..	2
20	6	9.8	1	4	1
30	4	9.2	2	1	1
40	3	10.5	3	..	..
60	3	10.5	3	..	..

* — One died 2 minutes after injection.

Subcutaneous injection

A number of mice were arranged in different groups and each mouse received one subcutaneous injection of Alizarin Red S. The individual doses were 20, 30, 40, and 60 mg/kg respectively. The same zones were colored as by intravenous injection. The results are reported in table 1C.

According to the results summarized in tables 1A and C, a single intravenous or subcutaneous injection of 20–30 mg./kg. of Alizarin Red S causes coloration of bone in 50% of growing

mice injected, and 50 mg./kg. gives coloration in all mice. In order to ascertain the time of occurrence of coloring after injection, 3 mice were injected with 60 mg./kg. of the dye and killed 2, 3, and 5 minues, respectively, after injection. The bones of these mice were found to be intensely colored. It was concluded that coloring takes place immediately after injection or resorption of the dye administered.

ADULT ANIMALS

Intravenous injection

A single intravenous injection of Alizarin Red S was given to different groups of mice in doses of 20, 30, 40, 50, 60, 80, 120, and 200 mg./kg., respectively. The results are reported in table 1B. These results show that the dose causing coloration in 50% of adult mice is 50–60 mg./kg. The same zones were colored as in the young animals, except that the coloration of the vertebrae was not diffused. The following protocol is characteristic for an adult mouse injected with 120 mg./kg. of the dye.

Coloration was found in the following zones: the premaxillaries; the malar process and around the alveoli of the maxillary (the alveolar wall was more intensely colored); the ethmoid; the bulla tympanica; the condyloid proces of the mandible (intensely colored) the mandibular notch; the mandibular symphysis; the alveolar part of the incisors; the occipital condyles; the crests and marginal parts of the parietal, interparietal, and squamous; the vertebrae — the bodies and the posterior articular, transverse and tubercular processes; the distal part of the caudal vertebrae; the coxal bone — the iliac crest, the inferior ventral spine, the articular surface of the sacrum, the acetabulum, and the ascending ramus pubis on the medial margin; the scapula — the vertebral border and the coracoid process (less intensively); the femur — the linea aspera, the third trochanter and the epiphyses around the condyle; the humerus — the epiphyses and the deltoid tuberosity; the ulna — the olecranon and the epiphyses; the epiphyses on

the tibia, fibula and radius; and the ribs—the sternal third part.

The pattern of coloration most frequently found corresponds to this description.

Subcutaneous injection

Six adult mice have been injected with doses of 80–120 mg./kg. Three showed slight coloration of the zones indicated.

Toxicological observations

During these experiments the possible toxic effects of Alizarin Red S (i.v.) were also watched. The reactions (dyspnea, jumping convulsions) were definitely not the same as the tetanic symptoms produced on injection of oxalate, which is known to reduce the concentration of circulating calcium ions. Three adult mice injected i.p. with doses of 500 mg./kg. of Alizarin Red S gave no tetany. They died 8–10 hours after injection, showing muscular weakness and dyspnea. It is concluded that Alizarin Red S does not bind circulatory calcium.

DISCUSSION AND CONCLUSIONS

The experimental results reported here demonstrate that bones of adult animals take up the alizarin dye if the injected dose, i.e., the concentration reached in the blood, is sufficiently high. Fixation can be considered to follow the absorption immediately since it occurs in less than 2 minutes after intravenous injection. To obtain 50% coloration adult mice require two to three times as great a dose as growing mice. Further, it was found that a dose of 80–120 mg./kg. given to adult mice by subcutaneous injection has only a slight coloring effect, but produces definite coloration by intravenous injection. Thus, in order to obtain visible fixation in adult bone, the intravenous administration is more adequate since it results in a higher concentration of the dye in the blood stream.

In order to obtain coloration of adult bone a higher dye concentration is required, presumably since in the older ani-

mal certain conditions would tend to diminish the quantity of dye which could reach the inorganic material; viz.:

1. Lower vascularization — According to Strelzoff, in pigeons, the diameter of the Haversian canals diminishes from 0.114–0.027 mm. in the 2-day-old animal, to 0.015–0.003 mm. in the adult. According to Lexer ('03) upon aging the vessels of bone become relatively thinner and their number diminish.

2. Lower permeability of adult bone — The increase of the relative mineral content at the expense of organic material and water (Weidenreich, '30) evidently results in more compactness and less permeability.

The percentage solubility of calcium alizarate (0.02%), higher than that of calcium carbonate (0.0013%), speaks against the assumption that the coloring may be due to calcium alizarate formed by reaction between dye and circulating calcium. An anion forming a more soluble calcium salt than carbonate could not possibly precipitate the calcium present in serum. In fact, the toxicological data show that Alizarin Red S, unlike oxalate, does not reduce the concentration of Ca ions in circulation.

Previously reported experiments and the results of this study suggest the following hypothesis concerning the mechanism of fixation of alizarin dye in bone. The circulating dye in contact with calcium phosphate is adsorbed with liberation of phosphate ion. Consequently, the coloration is localized mainly around the Haversian canals (Strelzoff, 1873) where contact occurs between inorganic material and dye. The adsorption reaction is irreversible; therefore, once fixed, the dye remains in bone tissue until the latter is resorbed (Flourens, 1847). The higher vascularity and permeability of growing bone leads to an uptake of the alizarin dye with lower doses than is the case in the adult. The zones where the coloration is maximum are the same in young and adult animals. In these zones, vascularization is greatest and metabolic processes, no doubt, are most intense. Emphasis is placed on the consideration that the higher fixation of alizarin in young

bone is due to the anatomical setup and not to any specific chemical condition of the calcium.

A high degree of diffusibility, electronegativity, and the irreversibility of the adsorption product have been reported as necessary for the fixation of a substance in the inorganic material of bone (Ercoli, '38, '41). In the light of the aforementioned hypothesis, the role of these factors becomes obvious:

a. Diffusibility facilitates the penetration of the dye into the compact material of bone.

b. Electronegativity allows its adsorption, since bone calcium phosphate behaves as an electro-positive adsorbent.

c. Irreversibility of the (dye)-Ca-phosphate adsorption product makes the fixation permanent. Trypan blue, an electronegative dye which has been found to produce temporary coloring of growing bone (Blotevogel, '26), forms a reversible adsorption product.

The small solubility of the calcium salt of alizarin led to the belief that the coloration is due to the formation of Ca salt. The relative insolubility of the Ca salt, however, is an indirect factor since only anions giving slightly soluble calcium salts are irreversibly adsorbed by calcium phosphate.⁴

The following experimental data confirm the hypothesis that coloring of bone takes place by polar adsorption between pre-existing calcium phosphate and circulating dye, rather than by formation of calcium alizarate or any reaction with Ca ions:

1. In vitro the reaction between inorganic bone material and alizarin is a polar adsorption process, closely following the isotherm of Freundlich (Ercoli, '38);

2. As reported in the experimental part, adult bone may be stained with the dye;

3. The fast rate at which coloring sets in;

4. The absence of toxic reactions, such as would be indicative of the disappearance of circulating Ca after administration of alizarin sulfonate, shows that no precipitation of Ca ions occurs;

5. The disappearance of coloration after decalcification proves that the dye is attached to the inorganic material; while

6. The permanence of coloration after treatment with alkali and destruction of the organic matrix also shows that the dye is fixed in the inorganic material.

In conclusion, the fixation of alizarin in bone in the form of a product of adsorption with pre-existing calcium phosphate, precludes its use as an indicator of the age of the deposited calcium salt or of the biochemical conditions underlying calcification. Nevertheless, the value of alizarin for studies of ossification remains unchanged, if its use is limited to determining the addition of new substance to that existing at the moment of the injection, or to the measurement of zones lying between those colored by dye administered at intervals.

SUMMARY

The influence of age on the fixation of Alizarin Red S was studied.

It was found that a single intravenous injection of 20–30 mg./kg. of dye caused bone coloration in growing mice. Adult mice required two to three times this amount to produce coloration. Coloration was produced in bone immediately after intravenous injection.

The anatomical zones in which coloration takes place have been described.

It is concluded that fixation occurs by a process of adsorption between existing calcium phosphate and dye anions, rather than by the supposed formation of calcium alizarate or a reaction with free calcium ions.

The higher dosage required for coloration in adult mice depends on the anatomical and physical, not on the chemical, conditions of bone.

In adult mice, subcutaneous injection produced less coloring than intravenous injection.

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THE EFFECT OF HYPOPHYSIAL STALK TRANSECTION UPON GONADOTROPHIC FUNCTION IN THE GUINEA PIG¹

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ONE FIGURE

The possibility of regulation of hypophyseal gonadotrophic function by the hypothalamus was suggested by Camus and Roussy ('20). Stimulation of the hypothalamus (Haterius and Derbyshire, Jr., '37) and lesions therein (Bailey and Bremer, '21; Smith, '27; Biggart and Alexander, '39; Dey, Fisher, Berry and Ranson, '40; Dey, '41) have confirmed this earlier observation; however, the effector mechanism by which the hypothalamus influences the hypophysis has not been demonstrated satisfactorily. Transection of the hypophyseal stalk has been reported; some animals had normal sex cycles post-operatively while other animals displayed marked gonadotrophic dysfunction (Richter, '33; Keller and Hamilton, Jr., '37; Dempsey, '39; Dempsey and Uotila, '40).

Anatomical studies have failed to explain the varied effects found upon stalk transection. Nerve fibers passing from the hypothalamus to the anterior lobe of the hypophysis have been reported (Brooks and Gersh, '41), but the sparsity of such fibers and the fact that they are absent in large areas of the hypophysis suggests that the hypothalamus may exert its influence in some other manner. Moreover, recent investigation (Dey, '42) has presented evidence indicating that the median eminence may be an important integer in the mediation of gonadotrophic function.

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The present study was undertaken to study further the post-operative estrous cycle after transection of the hypophyseal stalk at different levels in an attempt to evaluate the importance of the median eminence.

METHOD

Twenty virgin female guinea pigs weighing 500 to 900 gm. were employed in this investigation. Before operation the animals were observed daily for at least 2 weeks in order to establish the normal sex cycle. At the time of operation, performed under aseptic conditions, the left half of the calvarium was removed and the stalk approached subtemporally; the operative field was kept as dry as possible by means of an aspirator. This approach enabled the operator to see clearly the greater extent of the upper part of the infundibulum, and various levels of transection by means of a tiny scalpel were attempted. After a postoperative period ranging from 42 to 126 days, during which time the animals were observed for estrous cycles, the guinea pigs were sacrificed. The brains were fixed with formalin, and serial sections were cut at 30 micra. Every fourth section was stained with cresyl violet and the next following section by the Van Gieson method. The ovaries were fixed with formol-Zenker's fluid, cut serially at 10 micra, and stained with hematoxylin and eosin.

RESULTS

On the basis of postoperative sex cycles the animals of this series have been considered under four groupings: (1) animals which had regular cycles; (2) those which had irregular cycles; (3) those in which the vagina remained constantly open; and (4) those in which the vagina remained constantly closed.

Group 1. Seven animals ran normal estrous cycles post-operatively. The ovaries of two representatives of this group have been examined, and in each case normal follicular development and several corpora lutea were observed.

Upon examination of the brains it was evident that complete transection was accomplished in only one animal. The level of

section in this case was high, separating most of the median eminence from the hypothalamus proper; however, a small dorsal strand in the caudal half of the stalk was left attached to the hypothalamus above the section. The level of this transection, as well as that of other high lesions, is indicated in figure 1. In three animals, only the most anterior portion of the median eminence was severed, leaving intact most of the median eminence and any lateral and caudal connections to or through it. In the remaining three, a partial, asymmetrical section of the stalk had occurred. The ventral surface of the hypothalamus was not injured in any of these animals.

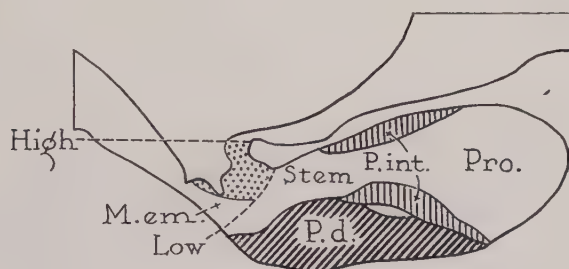


Fig. 1 Midsagittal reconstruction of representative high and low transections of the pituitary stalk. The broken lines indicate the levels of section. Region damaged in the diestrous animals is indicated by stippling. M. em., median eminence; P. d., pars distalis; P. int., pars intermedia; Pro., infundibular process; Stem, infundibular stem.

Group 2. Each of seven animals had irregular cycles in which the vagina was open most of the time. At sporadic intervals the vaginal membrane closed for a few days, only to rupture once more. The cystic, highly follicular condition of the ovaries of two representative animals suggested the secretion of excessive amounts of FSH. The presence of corpora lutea, however, indicated that ovulation had occurred, very likely just preceding the occasional closing of the vaginal membrane.

In one animal the median eminence was severed completely in its caudal portion, as shown in figure 1. In four, it was only partially separated on one side, thus leaving from one-half to

three-fourths of the median eminence connected with the hypothalamus. A high, incomplete section had been accomplished in one animal, leaving a small caudal strand of the infundibulum intact unilaterally. In the remaining animal of this group a high, complete section had been performed. This transection was quite similar to that in the single case of complete transection in group 1; however, the stalk was cut slightly higher at the caudal end, so that all of the median eminence was separated from the hypothalamus.

Group 3. The vagina of each of three animals was continually open during a postoperative period of at least 57 days. In a fourth case, the vagina remained closed for 61 postoperative days, then opened spontaneously and remained thus until the termination of the experiment 36 days later, at which time the external genitalia were hypertrophied, suggesting a hormone dysfunction similar to that of the other three animals of this group. The ovaries of a representative of this group were very cystic, with many large follicles. No corpora lutea were present.

In one animal a low, complete section in the caudal portion of the median eminence had been accomplished which left the greater portion of this structure attached to the hypothalamus. A higher section had been performed in the second animal of this group but left the median eminence intact laterally and caudally on one side. A moderately high, complete section in two animals separated the greater portion of the median eminence from the hypothalamus (fig. 1). In one of these animals a unilateral anemic softening was observed in a restricted region dorsolateral to the supraoptic nucleus. At its anterior margin this softening extended down to the ventral surface of the hypothalamus.

Group 4. The vagina of each of two animals remained constantly closed following the operation. The ovaries of one of the animals were examined and found to be atrophied. No corpora lutea or large follicles were present.

A high, complete transection had been effected in each of the animals, and a unilateral anemic softening similar to that

described above was present in one. A clear-cut difference between the transections in these two animals and the high transections described previously was the degree of damage to the median eminence itself in performing the transection. Approximately one-third to one-half of the caudal portion of this structure was damaged in each of the two diestrous animals. The level of section and the damage to the median eminence are shown in figure 1.

DISCUSSION

The animals which ran regular cycles after partial interruption of the infundibulum serve as excellent controls on the effect of the operative procedure excluding the actual transection of the stalk.

The high, complete transections of the hypophysial stalk found in all four groups suggest that there is no positive correlation between the level of transection and the resulting estrous cycles. The fact that normal estrous cycles may occur following stalk transection, as revealed in this and other investigations (Richter, '33; Dempsey, '39; Dempsey and Uotila, '40), does not favor the possibility of nerve fibers passing directly from the hypothalamus to the anterior lobe; for, if all of such fibers were interrupted by transection, one would expect an anestrus condition to follow. The possibility should therefore be considered of a hormonal rather than a neural activation of the cells of the pars distalis.

The role of the median eminence in gonadotrophic function is not yet apparent. Our data suggest that the amount of this structure severed from the hypothalamus is not important. The more extensive damage to the caudal portion of the median eminence in the two acyclic animals of group 4 may be of significance. These two animals sustained greater damage to this region than any other animals of the series, and they revealed a greater loss of gonadotrophic function. The anemic softening observed in the brain of the one animal is not considered to be pertinent in that the other animal was free of such injury. Our data focus attention upon the caudal median

eminence, but similar damage to the anterior portion may cause a like disturbance. It is suggested, therefore, that the varied effects of transection of the hypophysial stalk in spontaneously ovulating animals may be attributed to the amount of damage to the median eminence rather than to the level of the transection. Further investigation is necessary before this suggestion can be considered other than in the light of a possibility.

SUMMARY

Subtemporal transection of the hypophysial stalk was attempted in twenty virgin female guinea pigs. On the basis of postoperative sex cycles the animals were divided into four groups: seven animals had normal cycles; seven animals had irregular cycles; the vagina of each of four animals was constantly open; and the vagina of each of two animals was constantly closed.

The level and extent of transection were determined in each animal to see whether any correlation existed between the level of the transection and the resulting sex cycle. Since high transections of the infundibulum occurred in each of the four groups, it is concluded that a positive correlation does not exist.

A possible hypothalamico-hypophysial gonadotrophic interrelationship is considered, and the suggestion is made that the gonadotrophic dysfunction following transection of the hypophysial stalk may be attributed to the extent of damage to the median eminence rather than to the level of transection.

Grateful acknowledgment is made to Dr. H. W. Magoun for his surgical assistance in this investigation.

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GENITAL CHANGES IN FEMALE GUINEA PIGS RESULTING FROM DESTRUCTION OF THE MEDIAN EMINENCE¹

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ONE FIGURE

In a recent publication (Dey, '43) the effects of various hypothalamic and hypophysial lesions upon the gonadotrophic activity of the pituitary of the female guinea pig were reported. The results of this study indicated that the median eminence might be concerned in the regulation of pituitary gonadotrophic functions, insofar as lesions that encroached upon this structure abolished estrous cycles and caused genital atrophy.

In the present report, the effects of lesions placed directly within the median eminence are presented.

METHOD

Twenty-two mature female guinea pigs were utilized in this study. Trial operations were first performed in order to determine the Horsley-Clarke coordinates upon which the median eminence of the guinea pig lay. It was found that lesions located 8 to 9 mm. anterior to the interaural line and situated at the base of the brain would cause considerable damage to the median eminence. Lesions were placed in this region in the twenty-two experimental animals. In each animal three punctures were made in the brain, the first in the midline and the other two 1 mm. to either side of the midline. An electrode was lowered at each point until the base

¹ Aided by a grant from the Committee for Research in Problems of Sex, National Research Council.

of the brain was reached and the lesions were made by passing 3 milliamperes of current for 30 seconds. Lesions made in this manner usually fuse across the midline to form a single, large lesion.

Daily observations were made of the animals postoperatively and the condition of the vaginal membranes was recorded. The nature of the ovarian cycles was thus determined. The animals that ran normal cycles postoperatively were placed with males and observations were made for pregnancy. The animals that became acyclic were observed for at least 50 days and then the vaginal membranes were made to open by injecting the animals with ovarian hormones. The animals were injected with 100 i. u. of theelin² in four consecutive doses, on hours 0, 24, 48 and 60, followed by 0.2 i. u. of progesterone³ on hour 72. Following this artificial cycle, the animals were again observed for some 50 days before being sacrificed.

At the termination of the experiment the animals were killed by an overdosage of pentobarbital sodium and the brains and ovaries were prepared for histological study by our routine methods.

RESULTS

Of the twenty-two animals operated, seven ran normal cycles postoperatively. The fifteen remaining animals ceased running cycles and their genitalia atrophied. After the vaginal membranes of these fifteen acyclic animals had been closed for 50 days, they were opened artificially by the injection of ovarian hormones. The membranes remained open for a normal interval, 3 days, and then closed again. Eleven of the fifteen animals continued to be acyclic following this procedure, while the four remaining animals began to run regular, spontaneous cycles, and, after being placed with males, became pregnant and delivered normally.

² Received through the courtesy of the Parke, Davis & Company.

³ Received through the courtesy of the Schering Corporation.

The ovaries of the animals that ran normal cycles post-operatively and of those that regained normal cycles following the injection of ovarian hormones could not be distinguished from those of the normal animals. The ovaries of the permanently acyclic animals were markedly atrophic and lacking in follicular development. They resembled those found in the hypophysectomized animal.

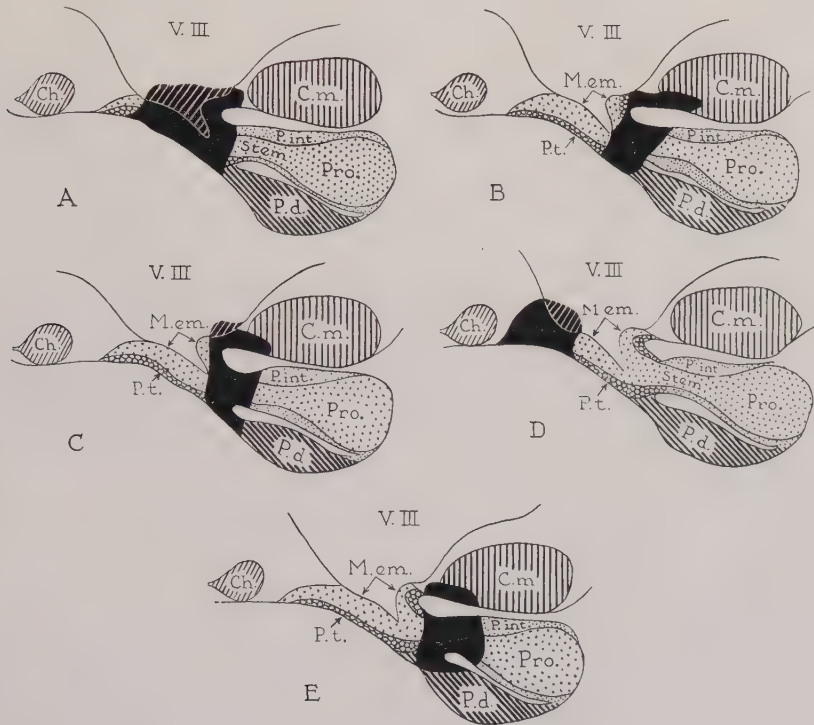


Fig. 1 Schematic reconstruction of mid-sagittal sections of the hypothalamus and hypophysis of the guinea pig upon which are projected various representative types of lesions encountered in this experiment. A, The lesions of the permanently acyclic animals, with one exception, caused extensive damage to the median eminence; and, B, the lesion of the exceptional animal. C, The lesions that only temporarily abolished estrous cycles caused but slight damage to the median eminence. D and E. Lesions that had no effect upon estrous cycles lay either rostral or caudal to the median eminence. Ch., optic chiasma; C. m., mammillary body; M. em., median eminence; P. d., pars distalis; P. int., pars intermedia; Pro., infundibular process; Stem, infundibular stem; V. III, third ventricle.

The brains of all the animals were examined microscopically. The lesions were found to vary somewhat in location within the various groups, but, in general, there was some correlation between the location of the lesion and the type of postoperative activity displayed by the animals. The lesions that permanently abolished estrous cycles, with one exception, were located within the median eminence and destroyed a major portion of that structure as shown in figure 1 A. The lesion of the exceptional animal completely severed the hypophyseal stalk, but caused only slight visible damage to the median eminence itself (fig. 1 B). The lesions of the four initially acyclic animals that regained cyclic activity following the injection of ovarian hormones were more caudally placed than those of the permanently acyclic group and consequently caused only slight damage to the median eminence. In three of the animals the main body of the lesion lay caudal to the median eminence and destroyed only a small portion of the caudal end of that structure (fig. 1 C), while in the fourth animal, nearly all the caudal half of the median eminence was destroyed. In each animal the lesion completely severed the hypophyseal stalk.

The lesions that had no effect upon the sex cycle lay either rostral (1 animal) or else caudal (6 animals) to the median eminence. The anterior lesion injured only the rostral tip of the median eminence as shown in figure 1 D, while the caudal lesions caused no injury at all to that structure although in the majority of cases they severed the hypophyseal stalk (fig. 1 E).

DISCUSSION

In previous investigations, we studied the effect of various hypothalamic and hypophyseal lesions upon the estrous cycles of female guinea pigs (Dey, '43). Genital atrophy and loss of ovarian cyclic activity occurred in only eleven animals although a large series of animals were studied. Information gained from these studies indicated that destruction of the median eminence or some closely related structure was re-

sponsible for the loss of estrous cycles that occurred in the few animals.

In the present experiments, we attempted to place lesions directly within the median eminence. The results of these experiments may be regarded as offering further support to the suggestion that the median eminence plays an important role in the control of the gonadotrophic functions of the anterior pituitary. Destruction of a major portion of the median eminence invariably caused a cessation of sex cycles.

Four of the fifteen acyclic animals of the present series regained normal cyclic activity following the administration of a "priming dose" of ovarian hormones. The damage to the median eminence in these four animals was considerably less than that seen in the eleven animals that remained permanently acyclic. The temporary loss of cycles in these animals might be explained as follows: The initial functional damage to the median eminence due to edema probably was much more extensive than that later shown in the microscopic sections. During this initial period of trauma, the hypophysis was deprived of whatever influence this area has upon it and failed to secrete gonadotrophic hormones. When the edema of the median eminence later subsided the body was depleted of ovarian hormones and consequently the initial stimulus for the release of gonadotrophic hormones by the pituitary was lacking. Therefore, normal cycles were not resumed. It is interesting to note that once ovarian hormones were supplied, normal cycles were immediately resumed and continued spontaneously until the animals were sacrificed a number of months later.

Dempsey ('39) demonstrated that normal sex cycles may occur in the female guinea pig following transection of the hypophysial stalk, and confirmation was provided by the findings of Leininger and Ranson ('43). Although normal cycles did occur in a large number of the animals of these series, yet both groups of investigators noted that a significant number of the experimental animals failed to run sex cycles following this procedure. It seems unlikely that the loss of sex

cycles in these cases was due to trauma to the pituitary gland since it has been our experience as well as that of Burch and co-workers ('37) that a large portion of the anterior lobe (up to two-thirds of the entire gland in our experiments) may be removed without abolishing sex cycles. It is likely that the loss of sex cycles that frequently occurs after transection of the pituitary stalk is due to injury to the median eminence.

SUMMARY

Lesions placed in the region of the median eminence abolished estrous cycles in fifteen of twenty-two female guinea pigs. Microscopic study of the brains showed that the major portion of the median eminence was destroyed in fourteen of the acyclic animals. The lesion of the exceptional animal transected the hypophyseal stalk but caused only slight injury to the median eminence itself. The lesions of the seven animals that ran normal cycles postoperatively lay either rostral or else caudal to the median eminence.

Four of the acyclic animals regained normal estrous cycles following the injection of a "priming dose" of ovarian hormones. The damage to the median eminence in these four animals was considerably less than that seen in the permanently acyclic group.

These results may be regarded as offering further support to the theory that the median eminence plays an important role in the control of the gonadotrophic functions of the anterior pituitary.

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EMBRYOLOGY IN WAR-TIME BRITAIN

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The study of embryology in Britain has necessarily been diminished by the war. Those human anatomists whose interests lay in the field of descriptive embryology are either carrying on their teaching with much reduced staffs, often under evacuation conditions, and with little time to spare for research, or they are in the medical branches of the Armed Services.

Those zoologists who were concerned with much of the experimental embryology are also in the Services or are engaged on research work of national importance in fields where their specialized knowledge of certain aspects of the basic sciences has been of significance. Departments, too, have suffered from direct enemy action and much valuable, some irreplaceable, material has been destroyed. Finally, the publication of work done is made much more difficult by necessary paper restrictions, diminished supply of material for block making and labor shortage in the printing and binding trades.

In spite of these difficulties, however, a number of investigators have managed to produce work of definite interest and importance and it is the intention of this article to draw attention to some of this work.

INVESTIGATIONS INTO DEVELOPMENT OF MONOTREMATA

Flynn and Hill ('42), in continuation of their investigations on the early development of the Monotremata, based on material which is unique and irreplaceable, have published a preliminary communication on the later stages of cleavage

and the formation of the primary germ layers. The most striking result of this investigation is the demonstration of a fundamental agreement in the mode of formation of the primary germ layers in the monotremes and marsupials, and it affords further and striking proof of the close phylogenetic relationship of the Ornithodelphian and Didelphian stocks. It is very fortunate that Prof. J. P. Hill, who has such a wide knowledge of early marsupial and monotreme development, should have been able to collaborate in this important investigation.

Among other interesting work on the descriptive embryology of the mammalia is a contribution by Amoroso, Griffith and Hamilton ('42) on the early development of the goat and one by Samuel and Hamilton ('42) on living eggs of the golden hamster, *Cricetus auratus*.

A number of early human embryos have been described by British embryologists in the past 3 years. Johnston ('40, '41) has given a full report on an early embryo (H. R. 1) which is at approximately the same stage of development as Grosser's H. Schm. 10. Dible and West ('41) have described a human ovum at the pre-villous stage which shows a number of interesting features. They also discuss the origin of the exocoelomic membrane, the yolk sac and the amnion. This ovum is a particularly valuable one but, unfortunately, no menstrual data were available so that its age can only be surmised. Its general stage of development, however, enables one to say that it cannot be much older than 12 days.

Odgers ('41) has given an account of a presomite human embryo, aged about 22 days, which possesses a number of interesting features, including a chorda canal. Gladstone ('41) and Hamilton ('42) have together given a full report on another presomite human embryo, of 20 to 23 days' age, with a chorda canal and a primitive streak. They have devoted special attention to the vascular system and their investigation throws new light on the early differentiation of the earliest generations of blood cells.

INTERESTING PAPERS FROM STRANGWAYS RESEARCH
LABORATORY

In the field of developmental mechanics Waddington ('42a), has published a paper on the results of translocations of the organizer in the gastrula of *Discoglossus*. In this investigation it was shown that translocated pieces of different regions of the organizer, in young-gastrulae of *D. pictus*, retained their original potentialities for gastrulation movements. These movements were such that only very incomplete mingling of regions was brought about which may account for the absence of any definite inductive interactions between different regions. The host "regulated" to form a complete embryo within which the graft lay as a more or less independent mass of tissue.

On the relationship of gene activity to embryonic development reference can be made to the work of Lees and Waddington ('42) on the development of bristles in normal and mutant types of *Drosophila melanogaster*; of Waddington ('42 b and c) on body color genes and on pupal contraction as a possible "epigenetic crisis" in the development of *Drosophila*: and of Pilkington ('42) on the structure and development of the compound eyes in normal and facet-mutant varieties of *D. melanogaster*.

Several interesting papers have appeared from the Strangeways Research Laboratory, in continuation of the work of Miss Fell and her collaborators, on bone development and growth. Jacobson and Fell ('41) have investigated the origin and early development of the muscles, cartilage and bone of the embryonic chick mandible. The technique employed was explantation of small portions of different areas of the jaw region in vitro with careful histological control of the precise site of origin of the explant. The subsequent differentiation of the explants appears to demonstrate that the myogenic cells migrate into the mandibular arch, as a histologically distinct group, between the second and third days, and that they are "determined" to form cross-striated muscle as early as the third day.

The chondrogenic cells originate from a small proliferation center immediately beneath the buccal epithelium of the branchial arch and they are "determined" to form cartilage in the 3-day jaw. The osteogenic cells destined to form the angular, surangular and splenial bones arise from a proliferation site which appears at the fourth day and which later divides, by cell degeneration, to form the rudiments of these three bones. The cells of the initial proliferation center are already "determined" to form bone in the 4-day mandible. The dentary arises a little later (fifth day) from a separate proliferation center and its cells are self-differentiating when explanted at the fifth day.

This contribution is of great interest and importance in the significant problem of early localization of differentiation in the embryos of higher vertebrates. Glucksman ('42), from the same laboratory, has studied the effects of mechanical stresses on cartilage and bone grown in vitro. His results demonstrate quite clearly that pressure and tension stresses exerted on cartilage in vitro cause a reorientation of the cells and lead to a disintegration of the hyaline matrix and its replacement by a fibrillar system. Tension stresses promote bone formation in osteogenic tissue in vitro and determine the pattern of the resulting osseous architecture. Glucksman's general conclusion is that skeletal tissue cultivated in vitro responds directly to mechanical stresses.

ASCORBIC ACID DISTRIBUTION IN CELLS AND TISSUES

Another interesting development is the work of Barnett and Bourne ('41, '42) who have studied the distribution of ascorbic acid in the cells and tissues of the developing chick embryo, basing their results on the acid nitrate technique. They give details for different tissues and organs and discuss the possible relationship of the ascorbic acid to intra-cellular organelles. They also discuss the possible role of ascorbic acid in developmental processes. This work opens up an important field of investigation and, doubtless, will be applied to the embryos of other animal groups.

Other contributions of interest to embryologists and foetal physiologists have appeared on the foetal circulation and deal particularly with the mechanisms for separation of the blood streams in the foetal right atrium, the ductus venosus and the closure of the foramen ovale and the ductus arteriosus (Franklin, Barclay and Prichard, '41; Amoroso, Barclay, Franklin and Prichard, '42; Amoroso, Franklin and Prichard, '42; and Boyd, '41).

Finally several books of considerable interest to embryologists have appeared in the course of the war years. de Beer ('40) has published, in new format and with an enlargement of the argument, a new edition of his well known "Embryology and Evolution." This little book had been out of print and, under its new title of "Embryos and Ancestors" it must be regarded as the soundest statement available on the relation of embryological fact to the theory of recapitulation.

Waddington's book, "Organizers and Genes" ('40), is one of the series of Cambridge Biological Studies and it represents a considerable contribution to the difficult problem of the relationships between gene activity, as demonstrated by modern genetic research, and the facts relating to organizers which have been revealed by the investigators of the causal mechanics of development.

Needham's eagerly anticipated "Biochemistry and Morphogenesis" has recently appeared (Cambridge University Press, '42). This large volume is in part a bringing up to date of the material which appeared in the author's "Chemical Embryology," but it is much more than this and represents a fundamental contribution to the theory of morphogenesis which can be neglected by no one interested in the processes which "mint and coin" the organism from the egg.

Sir D'Arcy Wentworth Thompson ('42) has again placed all biologists in his debt by the publication of a second edition of his "On Growth and Form." The first edition of this book appeared in 1916, during the first world war, and it was such a mine of precious information and such a helpful guide to action for biologists generally that it had long been out of

print and had, indeed, achieved the distinction of a real scarcity value. The new edition is slightly larger and is brought up to date, but it has not changed in spirit and represents the distillation of the life-long thoughts of one who is a classical scholar of distinction, a considerable mathematician and a great biologist. Here, indeed, as Needham puts it, is one of the golden books," invaluable to any embryologist.

Embryology in war-time Britain, like the island in which it is studied, is a beleaguered fortress in which principles have been kept inviolate and where interest in long-range projects has been kept active and alive by a much reduced group of investigators. Working under abnormal conditions and with many special duties (intensive teaching, A. R. P., First Aid, and Service in the front line, at home and abroad) these investigators have managed to produce a surprisingly large amount of work, only some of which could be referred to here.

They are symbolized by that embryologist who emerged from his blitzed house with a box of microscopic slides tucked under his arm!

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TWO CASES OF BILATERAL SUPERIOR VENAE CAVAE, ONE DRAINING A CLOSED CORONARY SINUS

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TWO FIGURES

Recently in this laboratory two cadavers were found to have bilateral superior venae cavae. One of these (case 1) is of particular interest because the left superior vena cava does not empty into the right atrium via the coronary sinus but receives blood from a closed coronary sinus.

Case 2 in addition to having bilateral superior venae cavae shows a true bilateral azygos venous system draining the thoracic walls.

ANATOMICAL DESCRIPTION

Case 1. The anomalous left superior vena cava follows the course of and replaces the oblique vein of Marshall. At the left extremity of the coronary sinus it lies behind the left auricular appendage, in front of the superior and inferior left pulmonary veins. Then it continues upward, curving laterally to describe an arc with its convexity toward the left, passes behind and then to the left of the common pulmonary artery and follows a groove medial to the left lung and pleura and antero-lateral to the aortic arch. By turning sharply to the right it terminates in the lower posterior part of the left innominate vein directly anterior to the beginning of the left subclavian artery.

The total length of the vessel is 11 cm. It receives the pericardiophrenic vein which, after running upward from the diaphragm, closely spirals round the left phrenic nerve and joins the left superior vena cava 1.2 cm. below the left innominate vein. The left superior intercostal vein drains the second, third, and fourth left interspaces and meets the left superior vena cava at a point 2.2 cm. below the left innominate vein. The first interspaces were drained by the venae supremae into the vertebral vein of the corresponding side.

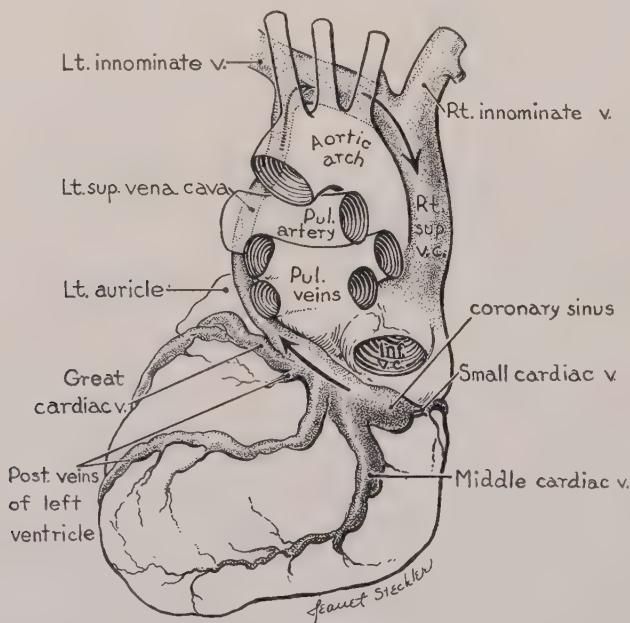


Fig. 1. Diagrammatic representation of blood flow from a closed coronary sinus through a left superior vena cava.

The coronary sinus receives the great cardiac and left marginal veins through a common opening while the middle cardiac vein, posterior vein of the left ventricle, and the lesser cardiac vein open into the sinus by means of separate ostia. No communication was found between the coronary sinus and the interior of the right atrium. The inner surface of the atrium is smooth except for the presence of a small unvalved

cul-de-sac 2 mm. in diameter and 1.5 mm. in depth which is the only evidence suggestive of an atrial opening of the sinus.

Case 2. The left superior vena cava is nearly as large as the one on the right. Its distal end is 1.6 cm. in diameter while

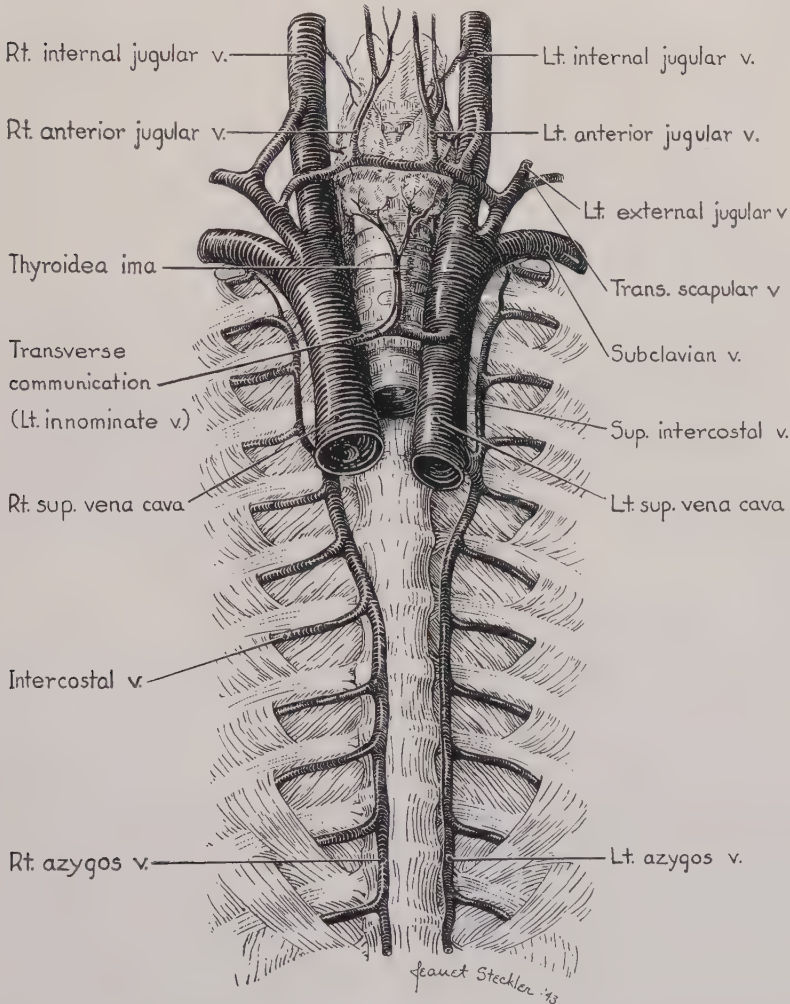


Fig. 2. Illustration of a complete bilateral venous azygos system and bilateral superior venae cavae.

the right one measures 2 cm. The diameters of the proximal ends vary even less; the left one being 1.4 cm., the right 1.5 cm. The left superior vena cava is 12.5 cm. long, and the right superior vena cava is only 9.5 cm. in length.

The relations of this vena cava are similar to those in case 1 except that the vena cava of this cadaver follows a less tortuous, practically perpendicular course.

Blood from the left subclavian vein, and from the left external and internal jugular veins was conveyed downward in the left superior vena cava, through the coronary sinus, into the right atrium. The ostium of the coronary sinus opening into the right atrium is 3.7 cm. in diameter and possesses no Thebesian valve.

The left internal mammary vein and a left azygos vein empty into the abnormal vena cava, the left azygos vein joining the caval trunk 6.3 cm. below its formation.

On the right side the superior vena cava is formed principally by the right innominate vein, but it received some blood from the left superior vena cava through a small transverse communication (the left innominate) averaging 0.32 cm. in diameter. It also received blood from the right internal mammary and right azygos veins.

The azygos veins are bilaterally symmetrical. The right one is normal in position and drained the usual interspaces. The left azygos vein is an exact counterpart of the right one. These azygos veins do not communicate with one another but empty into the superior venae cavae of their respective sides.

REVIEW OF LITERATURE AND DISCUSSION

Case 1. Double superior venae cavae are not infrequent. Chouke ('39) reported one case and found 205 cases previously described. However, the occurrence of a persistent left superior vena cava draining a closed coronary sinus is very uncommon. Up to this time only ten cases have been recorded. Le Cat (1738) is credited with the first report of this type of anomaly.

Gruber (1885) and Siding (1896) described adult hearts with closed sinuses which ended short of their normal openings into the right atrium. The inner wall of the right atria in both cases showed a small blind pouch which was guarded by a Thebesian valve.

Nabarro ('02), Beyerlein ('14), Hutton ('15), J. H. Smith ('19), Harris, Gray and Whitney ('27) and Reed ('38) also described closed sinuses. Peele ('32) found a case of bilateral superior venae cavae and a coronary sinus which was closed except for a minute channel connecting the sinus to the right atrium, which was too small to transmit any large amount of blood.

This case is the eleventh of its kind to be reported.

Case 2. The coronary sinus which appears to be the greatly dilated terminal end of the superior vena cava in case 2, measured 5.6 cm. in length and at its most dilated part is 3.2 cm. in width. Walsham (1880) suggested a reason for the tremendous dilatation of the left superior vena cava at the coronary sinus and a reason for the huge opening into the right atrium. He stated that the vessel probably had yielded to the backward pressure of the blood during auricular systole. Such yielding could take place because distally the vena cava consists mainly of the coronary sinus, and by being thin-walled and provided with a delicate valve is not able to withstand the stress exerted on its walls at each auricular contraction. It is probable that the Thebesian valve in this specimen atrophied from disuse when large amounts of blood passed through the vena cava; and absence of the valve in connection with the thinner wall of the distal portion of the vena cava would account for the dilatation.

Although more than 200 instances of abnormal venae cavae have been reported, few authors have adequately discussed the venous drainage of the chest walls in relation to the anomalous vessels they described.

Peele ('32) and Papez ('38), in their reports mentioned left superior venae cavae which received the accessory hemiazygos vein on the left, the azygos on the right being normal.

Keyes and Keyes ('25) and Chase ('38) found bilateral superior venae cavae with reversed azygos venous systems. The azygos major was formed on the left and received the accessory hemiazygos and hemiazygos veins from the right.

Of twenty-three authors who described cases of situs inversus of the superior venae cavae, Charles (1889), Atwell ('38) and Halpert ('42) reported the reversal of the azygos venous system and of a left azygos vein emptying into the left superior vena cava.

Five occurrences of bilateral venae cavae with duplicature of the azygos veins have been cited by Walsham (1880), Howden (1886), McCotter ('16), Huffmire and Bower ('19) and Chouke ('39). These workers described azygos veins on the right and left arching upward, passing behind and then above the roots of the lungs to join the vena cava superior of the right and left side respectively.

W. C. Smith ('16) reported a unilateral arrangement of the superior venae cavae but bilateral arrangement of the azygos veins. The left azygos flowed into the left superior vena cava and the right azygos entered the right innominate vein.

SUMMARY

Two cases of persistent left superior venae cavae are presented. The first case, the eleventh to be reported, demonstrates a closed coronary sinus necessitating a reversal of blood flow. The second case presents an anomalous left superior vena cava emptying into the right atrium through an extra large opening, and a complete bisymmetrical azygos venous system.

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THE COMPATIBILITY OF RAT AND MOUSE CELLS IN MIXED TISSUE CULTURES ¹

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INTRODUCTION

Transplantation of tissues and organs between different species of higher animals may succeed in embryonic hosts (Murphy, '26), but similar combinations are not compatible in adult stages (Loeb, '30). Thus while both normal and tumor tissues from rats and mice may be cultivated on the chorioallantoic membrane of young chick embryos (Murphy, '12, '13, '14a), and even human skin will proliferate in this environment (Goodpasture, Douglas, and Anderson, '38), such grafts are only temporarily successful and regress if maintained in hosts beyond the eighteenth day of incubation (Murphy, '14b). Interspecific transplants in adult birds and particularly in mammals are rapidly destroyed (Loeb, '30). Regression of heteroplastic grafts in adult mammals is due to the development of antagonistic responses to the engrafted tissues, as shown by the studies of Loeb and Addison ('09, '11), Loeb ('20, '21), Loeb and Harter ('26), Fleisher ('21, '22), and others. These reactions involve humoral factors as well as the connective tissue, lymphocytes, and polymorphonuclear leucocytes of the host. Much of the work to date on antagonism in heteroplastic grafting is concerned with describing the detailed pattern of the host response under conditions where the species used as host or the type of tissue transplanted varied.

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The causative mechanisms of tissue reactions to alien grafts are less well known. This may be traced, in part, to the natural disadvantages of transplantation as a method of studying tissue compatibility. In the living animal, it is difficult or impossible to separate individual aspects of tissue reactions from the total host response. In particular, transplantation does not reveal whether the responses by connective tissue, lymphocytes, and leucocytes are due to an intrinsic incompatibility between the cells of host and graft, or whether cellular activity of this type is conditioned by more general reactions occurring in the body of the host as a whole. On the one hand, cellular antagonism may be due to a direct local reaction between alien cells or substances given off by these cells, and the surrounding tissues of the host. Alternatively, tissue reactions might conceivably arise not as local phenomena, but as the result of focal sensitization to the graft, spreading secondarily over the whole body. In this connection, it is of interest to know whether a direct incompatibility exists between isolated cells of two different mammalian species, or whether cells of the two forms, if removed from complicating factors present in the body as a whole, may grow freely in association with one another.

The present investigation was undertaken with the foregoing question in mind. Studies were carried out on mixed tissue cultures containing normal rat and mouse tissues explanted together. Particular attention was given to the pattern of outgrowth, cytological appearance, and cellular behavior in such cultures. These criteria were used as a measure of compatibility between isolated cells of the two species.

MATERIALS AND METHODS

Tissues to be explanted were removed aseptically from newborn rats and mice. Heart, spleen, and kidney were selected as representative tissue types. For culturing, the roller tube technique as developed by Gey ('33) and W. H. Lewis ('35) was followed throughout. As a routine, cultures were planted in chicken plasma and a supernatant fluid added. In some

series of experiments, this fluid consisted of rat serum, with or without the addition of chick embryonic extract. In other experiments a completely heterologous medium was employed, the details of which will be described subsequently. At intervals of 2 or 3 days the cultures were washed in Locke solution and fresh supernatant fluid added. The large size of the culture vessels obviated necessity for subculturing during culture periods ranging up to 36 days.

EXPERIMENTAL

Growth in a heterologous medium

To rule out any possible influence of specific factors in the serum on tissue antagonism, a number of cultures were made in a medium entirely foreign to both rat and mouse cells. The fluid phase included chick embryo extract, Locke solution, and human cord serum (from placental blood obtained after birth), in a 1:3:3 ratio.

In an initial group of twenty-three experiments, fragments of rat and mouse heart were explanted together. The paired explants were placed in contact, closely adjacent, or at some distance from one another in the plasma clot, several pairs of explants being placed in each roller tube. Control cultures contained only mouse or rat heart, also in paired arrangement. During the first few days of culture in both experimental and control tubes, numbers of cells migrated outward from the explants, forming well-developed growth zones around the central fragments. Peripherally, these zones consisted primarily of mesothelium, with macrophages and fibroblasts forming the bulk of the remainder. In experimental cultures where mouse and rat explants developed adjacent to each other, the inner margins of the two growth zones came into contact. Among this line, rat and mouse fibroblasts, macrophages, and mesothelial cells intermingled freely. On subsequent days, outward migration continued from the two explants, resulting in an intimate interweaving of mouse and rat cells. In this way, the growth zones around adjacent rat and mouse explants fused smoothly without any clear line

of demarcation between the two types of cells. There was no evidence of abnormal cellular accumulation or reaction along the line of junction between the two explants. Fibroblasts, as well as the other elements present, appeared completely indifferent to the foreign cells, even in close proximity. Except for progressive growth, the composite structure formed by the fused explants was maintained without change during culture periods of 21-36 days. The central portions of the two heart fragments continued to pulsate for 2 or 3 weeks, although independently of one another in all cultures. Such composite cultures did not differ in degree of growth or cytological normality from control cultures containing paired fragments of rat or mouse heart only.

In another series of experiments, pieces of mouse spleen were explanted in association with rat spleen. Both were also cultured in separate tubes as a control procedure. In twenty-eight cultures of the experimental type, polymorphonuclear leucocytes, lymphocytes, and macrophages moved out in great numbers from the central explants during the first 24 hours of culture. Between adjacent explants, the amoeboid activity of these elements resulted in a mixed zone of outwandering cells from the two species. No sign of antagonism was apparent in such random aggregations of rat and mouse cells. Outward migration was approximately equal on all sides of the central explants, and the subsequent movements of the cells were apparently uninfluenced by the nearby alien tissue. In particular, these elements showed no tendency *in vitro* to surround or react to cells of the opposite species, or to move specifically in the direction of the foreign explant. The survival period of lymphocytes and polymorphonuclear leucocytes in both experimental and control cultures was limited to 48 or 72 hours. After this time, the growth zones around splenic explants came to consist of fibroblasts, macrophages, and mesothelium. Between nearby rat and mouse fragments, these peripheral regions united in a structure similar to that described for heart explants. Both rat and mouse cells re-

mained normal in appearance and growth continued until the experiments were terminated at 21 days.

Rat spleen and mouse kidney, or mouse spleen and rat kidney, were cultured together in a final group of twenty-two experiments. Similar results were obtained with either combination. Appropriate control cultures were set up containing kidney and splenic explants, both from the same species. The growth of kidney explants was variable, but large sheets of renal epithelium appeared around a number of fragments within 24-48 hours of culture. Where rat and mouse fragments had been placed close together, lymphocytes, polymorphonuclear leucocytes, and macrophages were frequently present near the kidney cells in great number. These foreign cells, however, did not appear incompatible in any way with the sheets of renal epithelium, and their presence near kidney explants resulted from normal outward migration and the proximity of the two explants, rather than specific accumulation in the vicinity of the alien tissue.

Kidney epithelium in both experimental and control cultures was overgrown and replaced after several days with fibroblasts and macrophages originating in the same explant. A connection was subsequently established between the growth zones surrounding kidney and splenic fragments. No further significant changes occurred over a 21-day period.

Growth in rat serum

In a parallel group of experiments, rat serum was substituted as a supernatant fluid in cultures, in place of a completely heterologous medium. Thus the rat cells were growing in a homologous serum, while mouse explants were exposed to an alien environment as in the foregoing experiments. This provided a means of testing whether tissue incompatibility depends on interaction of cells with a foreign serum, that is, whether an antagonism would develop against mouse cells in these mixed cultures if rat serum were present.

The specific combinations of rat and mouse tissues in these cultures were identical with those employed in the first group

of experiments, namely, paired explants of rat and mouse heart or spleen, together with a third series containing rat spleen and mouse kidney. The results of these various experiments may be treated together in a general description. In nearly all cultures both rat and mouse explants showed slightly better growth than in human cord serum and Locke solution; the effect was somewhat more marked on rat cells. This harmonizes with the finding of Lambert and Hanes ('11a) that nearly all mammalian sera exercise a slight inhibiting effect on alien cells in tissue culture.

Aside from minor effects on growth, the sequence of events in these cultures followed closely the pattern described for corresponding cultures in the completely heterologous medium. Outgrowths from rat and mouse fragments eventually united whenever the explants were sufficiently close for this to occur. Mouse cells in such cultures were invariably in good condition and showed no difference in cytological appearance from controls. During a culture interval of 21 days, there was no evidence of overgrowth or cellular antagonism by rat cells in the cultures.

DISCUSSION

These experiments demonstrate a complete indifference between rat and mouse cells maintained in close association in tissue cultures over a period of several weeks. Isolated cells from the two species are clearly compatible, therefore, as evidenced by the normal migration, growth, and appearance of individual cells in these cultures. It is of interest to compare these results with the descriptions of cellular phenomena in or around heterotransplanted mammalian tissue *in vivo*, as summarized by Loeb ('30). A marked lymphocytic reaction against heterografts does not develop for a week or more; this harmonizes well with the present experiments, where lymphocytes during their survival period of 48-72 hours in cultures, showed no cytotoxicity for foreign cells. Polymorphonuclear leucocytes, on the other hand, often accumulate around heterotransplants soon after operation: there was

no evidence of similar activity in tissue cultures. The behavior of fibroblasts in mixed cultures also contrasts strongly with their reported activity in the vicinity of foreign cells *in vivo*. In the body, this activity includes abnormal accumulation of fibroblasts near the foreign graft, as well as processes of encapsulation and infiltration. Such activity in the neighborhood of alien cells did not occur in any cultures in the present experiments. Even when directly in contact in culture, rat and mouse fibroblasts appeared entirely independent of, and unaffected by the foreign cells present. It is especially important that toxic phenomena, which play a major role in the destruction of heterotransplants in the body, were completely lacking *in vitro*. During culture periods up to 36 days, rat and mouse cells in mixed cultures were uniformly healthy and could not be distinguished from similar cells in control cultures containing tissue from one species only.

These findings are significant in their bearing on Loeb's concept of tissue antagonism, as set forth in his theory of organismal differentials ('30). Loeb assumes that cells in an alien environment give off factors, which either immediately or after direct interaction with the foreign body fluids, become toxic. In heterotransplantation these injurious factors, or heterotoxins, are themselves responsible for much damage to the graft, and further, serve to attract the lymphocytes, polymorphonuclear leucocytes and connective tissue of the host. These toxins initiate the antagonistic reactions of the host, whether in homoplastic or heteroplastic transplantation, and thus assume a central importance in the theory. The formation of toxins is conceived to be a local affair occurring directly between the transplant and body fluids surrounding it; to immune reactions Loeb attributes a secondary or complementary influence.

If tissue antagonism originates from a direct toxic reaction by cells in a foreign medium, it is of considerable interest that tissues may be successfully cultivated in alien sera, as shown by Lambert and Hanes ('11a) and many other workers. In discussing these results Loeb has pointed out that in ordinary

tissue culture technique the explant is surrounded by a relatively small amount of non-circulating fluid. Under these conditions the supply of toxins acting on the explant is assumed to be locally exhausted. The absence of host tissues in such cultures is mentioned as a further complicating factor. These objections, however, do not apply to the present experiments. The roller tube technique as used here has the dual advantage of using a relatively large volume of fluid medium which is kept in constant circulation by rotation of the tubes. In this way a fresh supply of serum is continually acting on the explant, and any toxic factors produced are circulated throughout the medium. Furthermore, these experiments have demonstrated that mouse cells grow normally in roller tube cultures containing both rat serum and actively metabolizing rat cells, the latter corresponding to "host" tissue. Under these circumstances there was no sign of toxic factors, either acting directly on mouse cells, or serving to attract rat fibroblasts.

It is difficult to harmonize these results with the concept that sensitizing toxins are produced by a local interaction between graft and foreign body fluids. It is easier to suggest tentatively that sensitization may be set up by central agencies in the body of the host against foreign proteins, or organismal differentials from the graft. Sensitizing factors could then spread through the body and while not acting directly on the graft, could modify the behavior of host tissues toward the implant. That an animal previously inoculated with an alien graft does exhibit an altered reaction to repeated transplants of the same tissue is evident, if the tissue used has a high growth energy, as for example tumor or embryonic tissue. It is a familiar fact that a number of tumors will grow for a time in alien hosts but later regress, leaving the animal immune from the growth of subsequent transplants of the tumor. M. R. Lewis ('40) has shown further that the development of such immunity, in the case of certain mouse tumors, occurs well before regression of the primary transplant sets in. Likewise the experiments of Rous ('10) indicate that mouse embryonic tissue, inoculated

into adult mice as a primary graft, will grow vigorously for a short time, although retrogression eventually occurs. A second injection of embryonic tissue in the same mouse does not grow at all. On the other hand, Loeb ('18) found that repeated transplantation of thyroid in rats failed to accelerate tissue responses to the grafts, and on this basis concluded that previous inoculation does not sensitize the host. Thyroid, however, is characterized by a low growth energy, especially in an unfavorable environment, and this disadvantage might obscure any differences between primary and secondary implants. Moreover, in determining whether or not the host has been sensitized by a previous inoculation, the growth or failure to grow by transplanted tissues possessing a strong growth potential would seem to be a more reliable criterion than the time relationships of host responses.

Further experiments are necessary to evaluate the foregoing hypothesis more completely, as related to the cellular phenomena in tissue reactions. Direct toxic action on heterografts may be ascribed at least in part to other, typical immune reactions. Lambert and Hanes ('11b), Lumsden ('28), the writer³, and others have demonstrated that sera from animals bearing heterotransplanted tumors are directly toxic to the corresponding cells in cultures. Since similar cytotoxins are not developed in mixed cultures of rat and mouse cells, it is probable that they do not form locally, but in a manner analogous to other typical antibodies.

SUMMARY

1. Paired rat and mouse tissue fragments have been cultured together in roller tubes. Heart, spleen and kidney from the two species were explanted in varying combinations.

2. In all cultures, adjacent rat and mouse fragments through cellular outgrowth eventually fused together to form a single, composite structure, stable over culture periods ranging up to 36 days.

³ Unpublished data.

3. Fibroblasts, lymphocytes, and polymorphonuclear leucocytes of both species migrated out normally from explants, without any attraction toward the foreign cells present. No evidence of encapsulation or specific accumulation in the vicinity of alien elements was observed.

4. Rat and mouse cells apparently are physiologically compatible in vitro. Toxic phenomena were completely absent in mixed cultures and both rat and mouse cells remained normal in cytological appearance.

5. The bearing of these results on current concepts of antagonism to transplanted tissues is discussed.

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REGENERATION OF THE LATERAL LINE NERVE OF AMBLYSTOMA FROM DIFFERENT NERVE FIBER SOURCES ¹

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ONE FIGURE

Past experiments on the volume of peripheral nerve fiber regeneration in amphibians have demonstrated that when the size of a nerve fiber source and the mass of its terminal tissues are varied independently, the fiber count in the regenerated nerve portion matches the latter rather than the former (Weiss, '37; Weiss and Walker, '34; Weiss and Litwiller, '37a, '37b; Litwiller, '38a, '38b). But the mechanism by which the periphery exerts this control over the density of its reinnervation is still obscure. Where fibers regenerate into a peripheral nerve stump, its complement of degenerated tubes (Buengner's cords) might be assumed to constitute the limiting factor. A peripheral stump after regeneration actually contains nearly its normal quota of fibers (Greenman, '13; Weiss, '37; Davenport, Chor and Dolkart, '37), just what might be expected if most tubes became reinvaded each by a single fiber. However, all tubes are not invaded; some tubes, in turn, receive more than one fiber, and some fibers grow in between tubes (Cajal, '28). Consequently, restoration of numerical order would appear to be more a matter of secondary adjustments than of limited penetration (Holmes and Young, '42). More facts are needed before the

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issue can be decided. Realizing that further analysis would be facilitated by the choice of a simpler, smaller and better accessible nerve than the limb nerves hitherto used, we have turned to the trunk lateral line nerve. Since many characteristics qualify this nerve to become a standard object in regeneration studies, we present the results of a preliminary survey of its regenerative capacities under various experimental conditions.

Large larvae of *Amblystoma tigrinum*, measuring from 3 to 6 cm. between fore and hind limb insertions, served for the experiments. The middle trunk line nerve, which runs antero-posteriorly in a canal under the flank skin, was used. For simple regeneration experiments, the nerve was transected about half-way between the fore and hind limb levels; in some cases, the peripheral stump was left in place, in others, it was pulled out. For regeneration from foreign fiber sources, the nerve was likewise transected in mid-trunk, but the proximal stump was removed and in its place a new fiber source was inserted facing the open end of the peripheral stump. The foreign innervation was provided by (1) the proximal stump of another lateral line nerve of either the same or the opposite side; (2) the deviated proximal stump of the hypoglossal nerve; (3) a transplanted spinal ganglion; (4) a transplanted fragment of spinal cord. Foreign nerves were hauled into the new site by means of a sewing needle through the eye of which they were threaded.

Some animals were sacrificed after 1 to 2 months, others only after 9 to 10 months. The nerves were studied in cross sections after paraffin embedding or in total mounts after dissection; all were impregnated with silver according to Bodian. Fiber counts in total mounts proved consistently too low because of fiber overlap; they require an upward correction of at least 20%.

Normal constitution of lateral line nerve. In animals of the size used, the nerve contains an average of fifty fibers (thirty-four counts in ten animals), falling in three size classes: small, medium, and large, in a numerical ratio of 1:2:1 (fig. 1). The

fiber number does not appreciably decline antero-posteriorly, which means that the many sense organs innervated by the nerve are supplied by collaterals of common fibers, a fact to be taken into account in the physiological interpretation of lateral line function. Preliminary observations on animals of different ages indicate that the majority of these fibers date back to the embryonic phase. Post-embryonically, the neurons increase but little in number though considerably in size (cf.

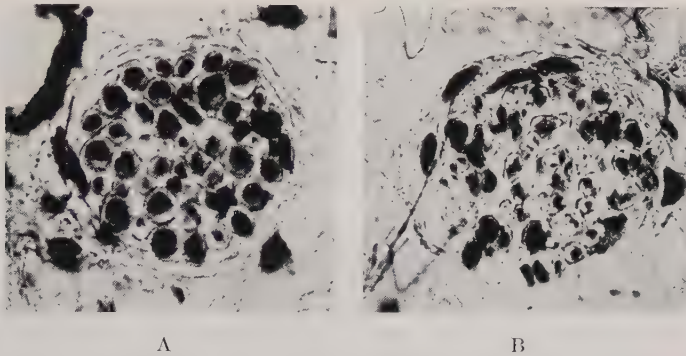


Fig. 1 Transected and regenerated lateral line nerve, 1 month after operation.

A, proximal stump B, distal (regenerated) portion. The large dark bodies in the regenerated nerve portion are nuclei. The young axons appear as small black dots, often several to a single old tube. $\times 500$.

Hursh, '39). On the whole, therefore, this nerve is sufficiently constant in its composition to serve as standard material for experimentation.

Transection with extirpation of peripheral stump. Fifty-three specimens subjected to this operation gave uniform results, namely, abortive regeneration. The outgrowing proximal fiber stumps never reached beyond the immediate vicinity of the cut end; they form small neuromas, the branches of which scatter in near-by muscle and connective tissue, without ever replenishing the lateral line canal. The result could perhaps be ascribed to the lack of an adequate number of peripheral sheath cells to support the growth of the nerve. It is noteworthy that the sensory lateral line fibers make

what appears microscopically as intimate terminal contact with muscle fibers.

Simple transection. Of nine cases in this group, two were studied in cross sections (early stages) and seven in whole mounts (terminal stages). In the latter, the peripheral stump was reinnervated to the extent of from eighteen to twenty-eight fibers, with an average of twenty-four. Allowing for undercounting in total mounts, the actual number would probably lie above thirty. Even though this value represents a deficit from the average of fifty fibers of normal nerve, it is still of the same order.

The two cross-sectioned cases illustrate regeneration 1 month after transection. The regenerated axons are immature, consisting of neurofibrillar bundles not yet identifiable as distinct nerve fibers. In one case (fig. 1), some ninety fibrillar units were counted near the cut, and about sixty-five farther distally. They were grouped into forty-three bundles, and this may well be an anticipation of the future fiber total. Eleven tubes were empty in the regenerated portion near the cut, but only three to four farther distally. The second case contained twenty-four fibrillar bundles.

Crossing of different lateral line nerves. Most cases of this series failed because the crossed nerve slipped back. The peripheral stump remained uninnervated, but persisted as such. Of a total of twenty cases, only three can be properly evaluated. In two, the smaller upper nerve (normal count in total mounts: ten to seventeen fibers) was connected with the peripheral middle nerve and regenerated twelve and fourteen fibers, respectively. In the third case, the larger nerve was connected with the peripheral stump of the smaller one and regenerated ten fibers. These values are consistently below those obtained in ordinary regeneration of the middle trunk nerve and could be ascribed, in the first two cases, to the undersized fiber source, and in the third, to the reduced size of the peripheral nerve stump.

Proximal hypoglossal connected with peripheral lateral line nerve. A sample count of a proximal hypoglossal nerve gave

over 300 fibers. This large nerve regenerated extensively, but only a very limited number of fibers found admission in the peripheral lateral line nerve. Of ten cases studied in whole mounts, five showed no connection between hypoglossal and lateral line nerve, evidently because the gap between the stumps was too wide. In the remaining five cases, the peripheral lateral line nerve contained 32, 40, 13, 36 and 23 fibers, respectively, which is again within the range observed after mere transection. Cross sections of an early regeneration stage contained ninety fibrillar units, grouped into forty-two bundles, near the base, declining to thirty-five units in twenty bundles farther distally. This also compares favorably with the early transection cases reported above. The motor fibers of the hypoglossal seem to have had no difficulty in regenerating into the purely sensory lateral line nerve.

Spinal ganglion transplants. In nine cases, a spinal ganglion was transplanted in front of the cut end of a distal lateral line nerve stump, the proximal portion having been removed. Five cases were discarded because of staining failure. In one case the ganglion had disappeared and the nerve contained no fibers. The three remaining cases (cross sections) showed the ganglion with numerous nerve cells intact and 22, 28 and 33 fibers, respectively, present in the lateral line nerve reinnervated from the ganglion.

Spinal cord transplants as regeneration source. In contrast to similar grafts to the dorsal fin (Weiss '40, '41), flank grafts rarely survive, presumably for mechanical reasons. Only in two out of nine cases, the graft was evident, and in these it had regenerated an average of twenty (cross section) and fifteen (total mount) fibers into the lateral line nerve.

Discussion. It is clear from the experiments that the number of fibers in the regenerated portion of the lateral line nerve is independent of the number of fibers produced by the proximal stump. The hypoglossal nerve and the spinal ganglion transplants provided a source several times larger than the regular lateral line nerve, without causing an increase in the yield of peripheral fibers. Similarly, a small lateral

line nerve stump, when supplied from a larger lateral line nerve, contains fewer fibers than would be regenerated in the latter's own stump. The number of regenerated fibers in the peripheral stump of the lateral line nerve tends to approach the full normal quota; it rarely reaches it, never exceeds it. Evidently, therefore, the size of the peripheral stump limits the volume of fiber regeneration. The lateral line nerve is a singularly favorable object to prove that the nerve itself, rather than the terminal receptor surface, constitutes the limiting factor, for, as was stated above, even in the normal nerve, the fiber number bears no relation to the number of end organs innervated. How the old nerve controls the quantity of regeneration, remains to be determined. The observed grouping during intermediate regeneration stages of fibrillar units into approximately as many bundles as the normal nerve has fibers, is suggestive. However, we do not know how this grouping comes about. While some old nerve tubes contain but a single bundle, others contain several, and still others, although a minority, remain empty.

In contrast to limb nerves, which in urodeles can regenerate to full length even after removal of the peripheral nerve stumps, no nerves in the lateral line canal have ever grown out for appreciable distances unless inside of an old peripheral stump. It is uncertain whether this failure is to be ascribed to inadequate sheath cell outgrowth or to immediate absorption of the fiber branches by the surrounding segmental muscle and connective tissue. Further experiments will have to decide these points, as well as the interesting question of restitution of fiber diameters after regeneration from diverse sources.

Summary. The small and relatively constant fiber complement of the urodele lateral line nerve recommends this nerve as standard object for nerve regeneration studies. The number of fibers regenerated in the peripheral stump stays within the normal range, even if excessive fiber sources (hypoglossal nerve, spinal ganglia) are made available. Thus, the peripheral rather than the central stump determines the volume

of regeneration, provided that the proximal fiber supply is adequate.

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SEX DIFFERENTIATION IN TWO ANDROGENETIC SALAMANDER LARVAE, TRITURUS PYRRHOGASTER

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FOUR TEXT FIGURES AND ONE PLATE (EIGHT FIGURES)

INTRODUCTION

In a recent paper (Kaylor, '40) dealing with experimental haploidy in salamanders, the writer reported that two advanced larvae were obtained which were not of the expected haploid chromosomal constitution. One larva was a mixture of haploid and diploid cells, and the other entirely triploid, on the basis of preliminary chromosome counts. Despite the fact that neither larva was haploid, there was good evidence that both had developed androgenetically, i.e. with only the paternal chromosomes.

It was intended that these two animals should be reared for breeding experiments, but when one, the triploid, died during metamorphosis when it left the water too soon, they were both preserved for histological study. The haploid-diploid larva was 17 weeks and the triploid 16½ weeks of age when preserved. Photographs and descriptions of both larvae are given in the preceeding paper ('40). Several controls of similar age were fixed at the same time.

The detailed nuclear conditions and the evidence for androgenesis in these two animals are presented in this paper, together with a description of the gonads. Both animals were found to be females. The significance of this fact is discussed in the light of recent observations on the mechanism of sex determination in salamanders.

All animals were fixed in Bouin's and sectioned at 12 micra from the tail forward. Sections were stained with Harris' acid hemalum and Orange G.

OBSERVATIONS

1. Chromosome numbers and nuclear size in the larvae

Chromosome numbers have been determined in a larger number of mitoses and from more widely separated parts of these larvae since the former report. The present studies support fully the previous conclusions. In the haploid-diploid

TABLE 1
Distribution of mitotic figures in the two larvae

	HAPLOID-DIPLOID LARVA			TRIPOID LARVA
	Haploid counts	Diploid counts	Total	Triploid counts
Epidermis	14	1	15	6
Skin glands	12	4	16	..
Connective tissue	3	2	5	2
Cartilage	1	10	11	..
Spinal cord	1	3	4	..
Heart	2	1	3	4
Gonads	4	1	5	2
Kidney	1	1	2	1 *
Gut	15	13	28	2 *
Liver	2	2	4	5
Peritoneum	..	..	..	1 *
Total			93	23

* More than 24 chromosomes

larva, the chromosomes could be counted with accuracy in ninety-three mitotic figures. Thirty-eight cells contained approximately twenty-four chromosomes, which is the normal diploid number for this species of newt. Fifty-five figures had approximately twelve chromosomes (seven with exactly twelve, and forty-eight with ten to twelve chromosomes). These counts were made in the various tissues and organs shown in table 1. There was no evidence of unilateral haploidy in this larva, as Griffiths ('41) found in larvae of mixed chromosomal constitution in his experiments.

When the triploid larva was sectioned, the internal organs were found to be surprisingly well preserved, with the exception of the central nervous system, the skin, and parts of the intestines. Various organs still contained a number of mitoses, a few of which could be analyzed for chromosome numbers. Twenty-three counts were made, nineteen of which contained from $28 +$ to 35 ± 1 chromosomes, while four mitoses contained more than twenty-four chromosomes. These mitotic figures were distributed over the organs and tissues noted in table 1.

In addition to the direct test of the chromosomal conditions in these two larvae, there is a secondary test of nuclear size. Camera lucida drawings were made of the nuclei in various parts of the two larvae and a normal diploid control (figs. 1-3). While the majority of nuclei in the epidermis of the haploid-diploid were smaller than controls, other organs contained a more even distribution of small nuclei and nuclei of the same size as diploids. The triploid nuclei are clearly larger than diploids, indicating the uniformly triploid condition of this larva.

2. Evidence for androgenetic development of the larvae

These larvae developed from eggs which were punctured shortly after fertilization and a small polar area of cytoplasm, including the female nucleus, sucked into a pipette. The eggs were left otherwise intact with only the male chromatin to influence development.

The evidence for removal of the female nucleus is based on the delayed development of the two eggs during blastula, gastrula, and neurula stages. Control eggs from which cortical cytoplasm was removed in the immediate vicinity of, but not including, the female maturation spindle, were never retarded in their development as compared to unoperated controls. This type of evidence, although indirect, is nevertheless reliable since all investigators, using a variety of species, report that delay is distinctly a characteristic of androgenetic development.

Despite the general reliability of retarded development as evidence for androgenesis, there is still the possibility that circumstances other than removal of the maternal chromatin could conceivably have caused the observed delay (see discussion).

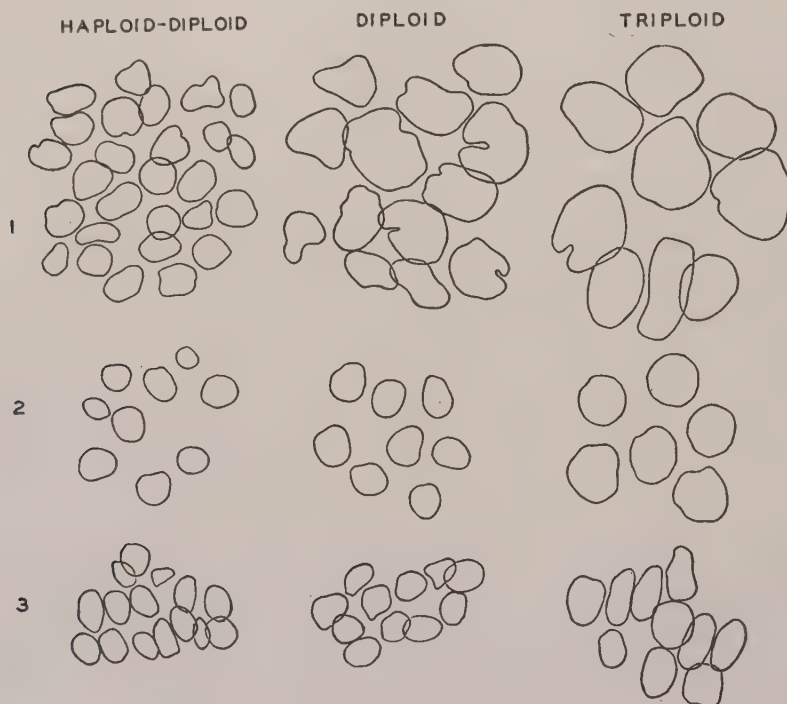


Fig. 1-3 Camera lucida drawings of nuclei from different regions of the haploid-diploid, a normal diploid control and the triploid larvae, 400 X. Figure 1, nuclei from a whole mount of the tail-tip of each larvae. Figure 2, nuclei in the liver of each larva. Figure 3, nuclei in the retina of each larva.

3. The gonads

a. Gonads in the controls. The normal process of sex differentiation has been studied in other species of salamanders by Abramowicz ('13, *Triton taeniatus*), Burns ('25, *Ambystoma punctatum*), McCurdy ('31, *Triturus torosus*), and by Fankhauser ('40, *Triturus viridescens*). All investigators

agree that at metamorphosis the larval gonads are clearly differentiated as either ovary or testis. The ovary possesses a small central cavity surrounded by oogonia and oocytes. The young testis, on the other hand, is a solid structure with germ cells distributed throughout the gonad. The spermatogonia have a lobulated nucleus in contrast to the spherical nucleus of the oogonia.

The gonads of several partially-metamorphosed normal *Triturus pyrrhogaster* larvae were studied. These animals ranged in age from 16½ to 18½ weeks. The normal gonad is well differentiated at this age, and an ovary in this species can be distinguished from a testis by the same features as have been described for other salamanders. The ovary (figs. 7 and 8) has a central cavity with oogonia at the periphery. The testis (figs. 5 and 6) in cross section is solidly packed with spermatogonia. As it happened, the ovaries of the two control females examined did not have the large oocytes which the closely related *Triturus viridescens* possesses at this time.

Normal sex differentiation in *Triturus pyrrhogaster* is, however, sometimes more difficult of interpretation than it was in the controls examined in these experiments. Often the gonads vary in degree of development, so that ovaries can not easily be distinguished from testes (Rita C. Watson, communication from Dr. G. Fankhauser).

b. Gonads in the androgenetic larvae. The haploid-diploid larva was fixed toward the end of metamorphosis, at an age of 17 weeks. A section through the largest part of the left gonad is shown in figure 9, and one through the right gonad in figure 10. Comparison of these two with corresponding sections through the control ovaries (figs. 7 and 8) shows that the haploid-diploid gonads, although unequally developed, are ovaries. A central cavity is present in each ovary and the germ cells with spherical nuclei surround the cavity. The right gonad is a normal ovary in all of its features, while the left is small and poorly developed. The latter, in cross section, is only about one half as large as the right and contains less than half as many germ cells. Size differences in the two

ovaries of the haploid-diploid larva are also clearly seen in the graphic reconstructions (fig. 4a).

The triploid larva was partially metamorphosed when it died accidentally and was fixed at an age of $16\frac{1}{2}$ weeks. Although the time elapsed between death and fixation of the animal is not known, sections through the gonads show them

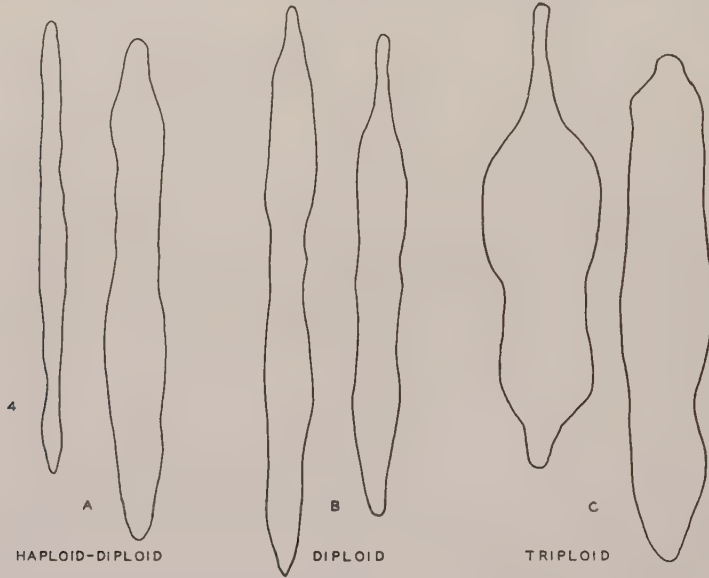


Fig. 4 Reconstructions of ovaries. A, haploid-diploid larva, 17 weeks. B, normal diploid control larva, $17\frac{1}{2}$ weeks. C, triploid larva $16\frac{1}{2}$ weeks.

Magnification of all gonads, $25\times$.

to be in a fairly good state of preservation, at least for the purposes of the present study. Representative sections through the left and right gonads are shown in figures 11 and 12 respectively. The central cavity and peripherally distributed germ cells leave no doubt as to the femaleness of these gonads. The most remarkable feature of these triploid ovaries is the extent to which they have developed beyond the ovaries of controls which had been raised and fed in the same manner as the androgenetic larvae (compare figs. 11 and 12 to figs. 7 and 8). The triploid ovary is several times the size of the

control ovary. The central cavity is more extensive and the germ cells more numerous in the triploid. The triploid germ cells have entered the growth period, i.e. the oocyte stage, as characterized by their size and the tortuous, spireme threads in the nuclei. Graphic reconstructions show the triploid ovaries (fig. 4c) to be three times the width of control ovaries (fig. 4b).

The proportions to which the ovaries developed in this triploid *Triturus pyrrhogaster* larva are in striking contrast to the stunted growth of ovaries in triploid *Triturus viridescens* larvae obtained in the experiments of Fankhauser ('40) and of Griffiths ('41). Confirmation of these observations has recently been received in the description of the gonads of two triploid *Triturus pyrrhogaster* obtained in cold experiments by Rita C. Watson (ref. cited above). The ovaries of these larvae were normally differentiated and slightly larger than in controls.

DISCUSSION

Two partly metamorphosed salamander larvae, one a haploid-diploid and the other a triploid, are described in this paper. The evidence presented indicates rather strongly that both larvae developed with only the male chromosomes. Direct proof for androgenesis is lacking since neither larva was uniformly haploid. This alone does not contradict the evidence for androgenesis, because the chromosomal conditions of the two larvae might be accounted for either on the basis of an early regulation from haploidy to haploidy-diploidy and to triploidy, or, in the case of the triploid, by a fusion of a haploid and a diploid sperm nucleus. The rather frequent occurrence of such phenomena in frog embryos (Parmenter, '33; Porter, '39) and in early androgenetic salamander eggs (Fankhauser, '34; Kaylor, '41) is certainly suggestive, and there is no reason why this should not have occurred in the two cases at hand. But there are exceptions to all rules, and it might still be claimed that circumstances other than the ones mentioned above may have accounted for the retarded

development and the unexpected chromosome numbers in these larvae.

Both of these larvae were females. The ovaries of the haploid-diploid were unequally differentiated—only one gonad was as large as the controls. The triploid gonads were well developed ovaries which were larger than normal. The present triploid larva and the two described by Watson (ref. cited previously) are the only triploid salamander larvae reported so far with normal ovaries.

The femaleness of both of these androgenetic salamander larvae is interesting in connection with the problem of sex determination in salamanders. Several spontaneous triploid larvae of *Triturus viridescens* were obtained by Fankhauser ('40) and a larger number of cold-induced triploids of the same species was reported by Griffiths ('41). The gonads of all of those triploid larvae were either normal testes or small, poorly developed ovaries. In animals in which the sex is determined by chromosomal distribution, it is well known that triploidy effects the gonads of the heterogametic sex. Therefore, Fankhauser's and Griffiths' observations indicate that the female of *Triturus viridescens* is heterogametic for sex. Recently, Humphrey ('42) has beautifully demonstrated female heterogamety in the axolotl, *Amblystoma mexicanum*. He was able to breed to normal females an axolotl female experimentally converted into a male and to determine the sex of offspring from such matings. The results of these different investigations suggest rather strongly, then, that the female salamander is heterozygous (ZW) for sex, while the male is homozygous (ZZ).

Assuming that these *Triturus pyrrhogaster* larvae developed with male chromosomes only, then if the male salamander were homozygous for sex (ZZ), both larvae should have been males: the haploid-diploid (Z)-(ZZ) and the triploid (ZZZ). If, on the other hand, the male salamander were heterozygous for sex (XY), as in *Drosophila* and others, then there was at least an even chance that these androgenetic larvae could have been females: (X)-(XX) and (XXX) female, or (Y)-(YY)

and (YYY) male (?). It is even possible that all androgenetic larvae should be females, if the mechanism is similar to *Drosophila*, for in the latter an embryo must have at least one (X) or female-determining chromosome for survival.

If by chance these larvae did not develop androgenetically, the female nucleus must have remained in the egg and as a result various combinations of paternal and maternal chromosomes are possible in explanation of the chromosomal conditions of the present larvae. As an example, taking only the present triploid larva, supposing a non-reduced female nucleus was fertilized by the haploid male nucleus. If the male is of the (ZZ) type for sex, then $(Z) \times (ZW) = (ZZW)$ intersex (?). If the male is of the (XY) type, then $(X) \times (XX) = (XXX)$ female, or $(Y) \times (XX) = (XXY)$ intersex (?). Considering other combinations such as these, the (XY) *Drosophila* type still offers the greatest number of possible explanations for the sex of these larvae.

The suggestion that *Triturus pyrrhogaster* may be of the (XY) type agrees with the observations on a haploid merogonic Triton larva (Fankhuaser, '38) which possessed well developed ovaries. However, Fankhauser's case has lost some of its weight with the discovery by Bööck ('40) in Sweden of an adult male triploid of the same species of Triton.

Whatever the mechanism may be in *Triturus pyrrhogaster*, the well developed ovaries of the present triploid larva, together with those reported by Watson, are in decided contrast to the abnormal ovaries in the other triploid species, and indicate that the mechanism for sex determination is not the same in this species as in *Triturus viridescens* and *Amblystoma*. It would not be surprising to find variation between species of salamanders, in view of the situation in fishes. Breeding experiments with hermaphroditic frogs (Witschi, '29) and with sex reversed toads (Harms, '26; Ponse, '30) indicate also that the mechanism in the anurans is variable. Full analysis of the problem of sex determination in amphibians will be complicated because zygotic determination is easily

influenced by many secondary factors (Humphrey, '29; Witschi, '36, '42).

SUMMARY

1. Chromosome counts and nuclear size determinations from various regions of two partly metamorphosed salamander larvae demonstrate that one larva was a mixture of haploid and diploid cells, and the other larva was entirely triploid.

2. The evidence presented suggests that the two larvae developed androgenetically.

3. The right gonad of one larva, the haploid-diploid, was a normally differentiated ovary. The left gonad of this larva was a poorly developed ovary.

4. The gonads of the triploid larva were well developed ovaries which were larger than control ovaries.

5. The significance of these findings is discussed in connection with the problem of sex determination in salamanders.

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PLATE 1

EXPLANATION OF FIGURES

All figures are photomicrographs of transverse sections through the gonads. Each figure is magnified $116\times$.

5 Left testis of normal control larva, $18\frac{1}{2}$ weeks. No central cavity, spermatogonia scattered throughout gonad.

6 Right testis of same normal control larva as in figure 5. Lobulated nuclei.

7 Left ovary of normal control larva, $18\frac{1}{2}$ weeks. Central cavity, peripherally arranged oogonia.

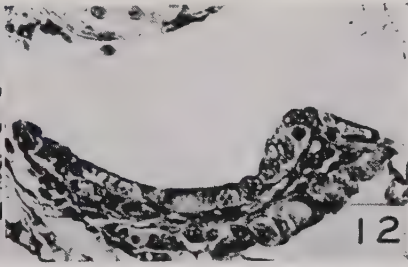
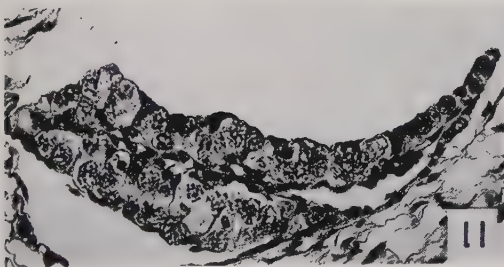
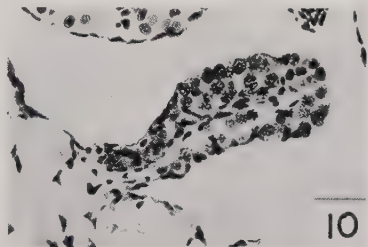
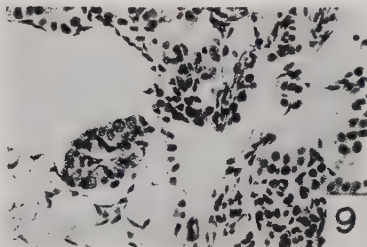
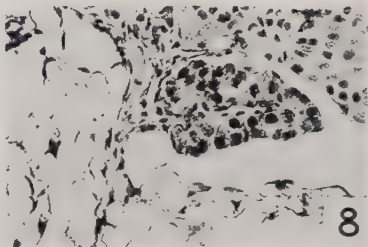
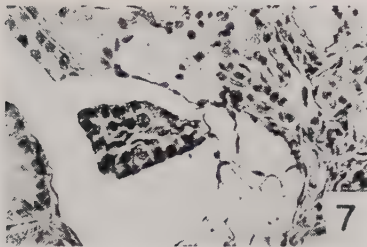
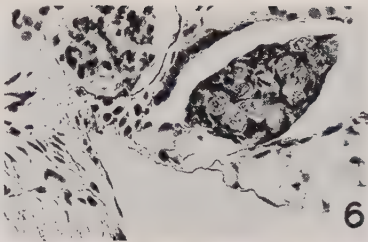
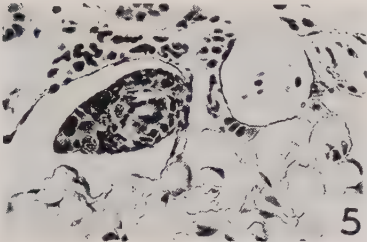
8 Right ovary of same normal control larva as in figure 7. Slightly larger gonad.

9 Left ovary of haploid-diploid larva, 17 weeks. Gonad reduced in size; central cavity small, germ cells peripheral.

10 Right ovary of haploid-diploid larva. Normal sized ovary; noticeable central cavity; nests of oogonia at periphery.

11 Left ovary of triploid larva, $16\frac{1}{2}$ weeks. Larger than control ovary; large central cavity, more advanced germ cells than in control.

12 Right ovary of triploid larva. Larger than control ovary.



GROWTH OF THE HUMAN SUPRARENAL GLANDS

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ONE FIGURE

Although it has been over 30 years since the neonatal loss in mass of the human suprarenal glands was described almost simultaneously by Thomas ('11), Kern ('11) and Elliott and Armour ('11), the functional significance of the involution and the function of the fetal cortex are still unknown. The inner fetal cortical tissue which undergoes degeneration was at first thought to be the zona reticularis of the adult cortex and similar to the remainder of the cortex. Elliott and Armour ('11) observed that the cells of the permanent cortex were smaller and darker than those of the fetal cortex. Keene and Hewer ('27) and Uotila ('40) found that the fetal cortex was formed from the columnar mesothelial cells at the 7- to 8-mm. stage while the cells of the permanent cortex formed from a further proliferation in the same area during the 11- to 13-mm. stage. Uotila observed that the fetal cortical cells were large and acidophilic while those of the permanent cortex were smaller and had smaller darker nuclei.

Attention was focused on the relationship of the adrenals to the sex glands by Bullock and Sequeira ('05) who described eleven cases of adrenal tumor in children which resulted in the development or accentuation of male secondary sex characters. Since that time abundant clinical evidence has been given which indicates that the suprarenal glands can produce

¹ Most of the data for this report was collected while the writer was a member of the faculty of the Medical College of the State of South Carolina.

androgenic substances. The experimental evidence regarding this point is controversial. Carnes ('40) was unable to extract androgenic substances from human fetal suprarenal glands. Regardless of whether the androgens result from normal or perverted metabolism of fetal cortical "rests" which failed to involute or result from normal or perverted metabolism of adult cortical cells, the adrenogenital syndrome in the adult (Virilism) and in children (Macrogenitosomia precox) has been reported to occur both with and without suprarenal enlargement. Normal cortical volume and corticomedullary ratios are available for the adult glands (Swinyard, '40); however, there are no quantitative data regarding the normal fetal cortex, permanent cortex and medulla of the suprarenal glands of fetuses, infants and children.

MATERIALS AND METHODS

From a collection of seventy-four pairs of glands from negro individuals ranging from $3\frac{1}{2}$ lunar months to 17 years of age, fourteen glands were selected for quantitative study. It is realized that the suprarenal gland is extremely variable and that the growth curve shown as figure 1, is based on relatively few glands. However, the glands selected for quantitative study were carefully selected from a larger series and are believed to be fully representative of a given age group. The labor involved in obtaining quantitative data of this type precludes the use of large numbers of glands. None of the specimens used had any pathological changes. The tissue was all collected within 24 hours after death, carefully cleaned of fat and extraneous connective tissue, and then weighed. The specific gravity of the fresh tissue was obtained by methods previously described (Swinyard, '39). The suprarenals were then fixed, dehydrated, embedded in paraffin and sectioned serially at 10 micra. Every tenth section was mounted and stained with hematoxylin and eosin. The sections were then projected with a Leitz Edinger projector and from the drawings made the volume of the gland and its subdivisions was obtained from planimetric measurement.

The figures obtained were corrected to the fresh volume to eliminate the effect of shrinkage. In the youngest fetuses considerable difficulty was encountered in measuring such diffuse small amounts of medullary tissue. Therefore, the volume of the medulla of these glands was also obtained with the paper weight method with very similar results.

DISCUSSION AND RESULTS

The cytology of the involutionary process has been described by Landau ('15) and Lewis and Pappenheimer ('16). They described the hyperemia of the inner cortical layer that loosened and displaced the cells which showed beginning degeneration in the first few days of postnatal life. Extensive degeneration was found at the end of the second week and was completely established at the end of the first month. From the sixth week to the end of the first year the stroma of the degenerating fetal cortex progressively narrowed. Lewis and Pappenheimer found no growth of the adult cortex during the first three years of postnatal life. They also observed normal involution in accessory cortical nodules which contained no medullary tissue. The involutionary process was not affected by prematurity, inanition or infection. Goldzeiher ('29) reported that the glands attained morphologic stability in the eighth year of life.

Scammon ('26) found no augmented rate of growth preceding the neonatal involution. The glands lost one third of the natal weight during the first 2 postnatal weeks and one half of the natal weight during the first trimester after birth.

Benner ('40) studied the normal range of variation during involution. She found that the suprarenal weights increased markedly as the fetal length approached 50 cm. Individual variation increased after the fetus reached 45 cm. in length. There was no evidence of sex variation in the glands studied. In the normal newborn Benner found either slight degeneration at the center of the gland or none at all. In 11% of the newborns there was a deviation from the normal which in most cases was a moderate increase in the amount of degener-

ation. Extensive degeneration began on the fourth postnatal day and was massive at the end of the second week. Both glands averaged 4.0 to 5.0 gm. in weight at the end of the second week and maintained this weight through the first year. Degeneration included four-fifths of the cortex by the beginning of the second week. By the third week the permanent cortex had increased in thickness two fold. In Benner's specimens which lived from 3 days to 1 month degeneration reached its greatest extent. There was continued sequestration of the fetal cortex in thirty-two infants ranging from 1 month to 1 year of age. There was neither evidence of a sex difference nor any correlation with pathological findings. In Benner's group of forty-four children ranging from 1 to 13 years of age there was progressive increase in medullary and adult cortical tissue.

In our specimens the medulla of the suprarenal glands of a 3½-month-old fetus was scattered in small clumps and constituted only a fraction of 1.0% of the total gland volume. The adult cortex comprised 15.3% of the total cortex the remainder of it being fetal cortex. The cortico-medullary ratio was 382.7:1 (table 1, fig. 1).

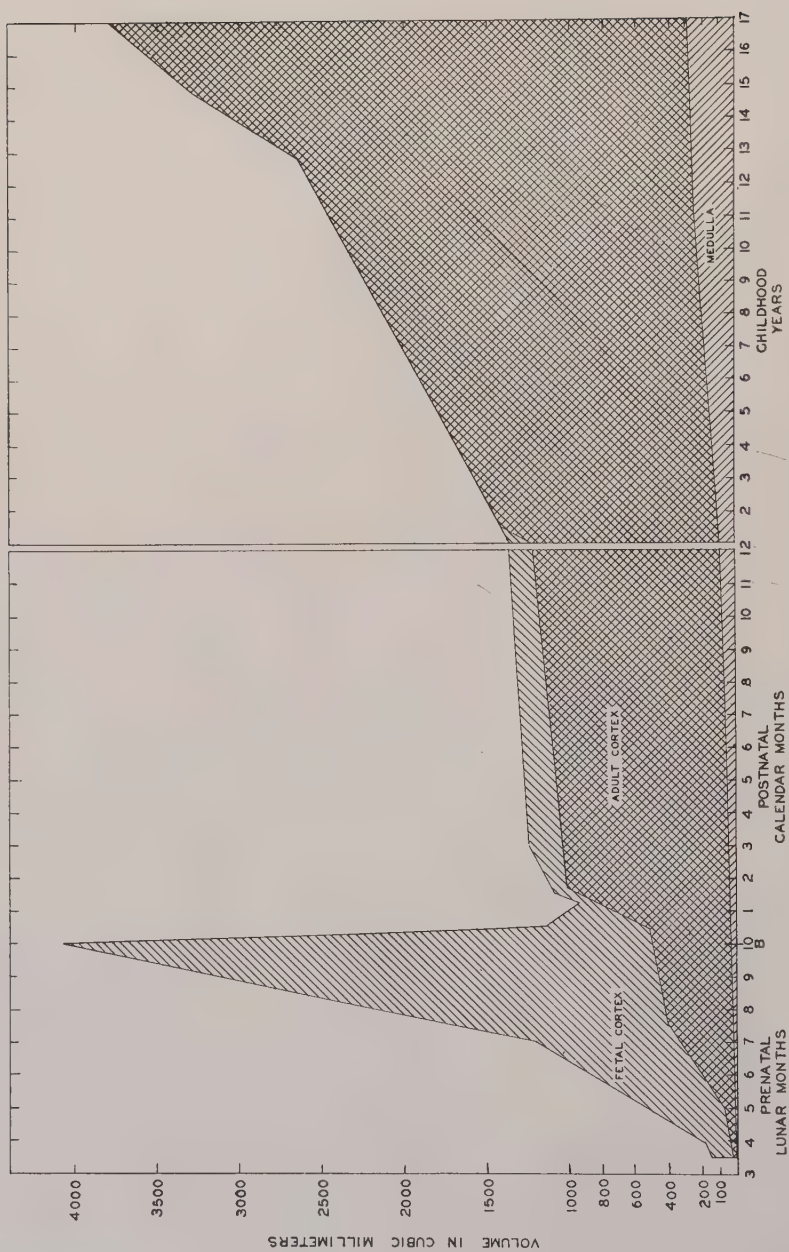
In a 5-month fetus the total volume of the gland had increased to 577.9 cu.mm. The proportions of fetal and adult cortex remained approximately the same, but the medulla had increased to a little over 2.0 cu.mm. The increased volume of the medulla reduced the cortico-medullary ratio to 279.5:1.

During the beginning of the third trimester of fetal life (6½ lunar months) both the cortex and medulla grew more rapidly. The total volume was 1310.2 cu.mm. of which the medulla constituted 1.1%. The adult cortex formed 37.6% of the total cortex. The increased medullary volume reduced the cortico-medullary ratio to 95.3:1.

During the last half of the third trimester of prenatal life the fetal cortex apparently grew much more rapidly than the adult cortex (fig. 1). This resulted in a high cortico-medullary ratio at birth (213.8:1). The rapid growth rate of fetal cortical tissue in late fetal life accounts for Benner's observation

TABLE 1
Volumetric comparison of the cortex and medulla of negro suprarenal glands at different prenatal and postnatal ages.

SEX	AGE	SUR- VIVAL TIME	TOTAL VOLUME CU. MM.	TOTAL CORTX VOLUME CU. MM.	ADULT CORTX VOLUME CU. MM.	FETAL CORTX VOLUME CU. MM.	MEDULLA VOLUME CU. MM.	ADULT CORTX IN % TOTAL CORTX	FETAL CORTX IN % TOTAL CORTX	ZONA GLOM- ERULOSA VOLUME CU. MM.	ZONA FASCIC- ULATA VOLUME CU. MM.	ZONA RETIC- ULARIS VOLUME CU. MM.	CORTICO- MEDULLARY RATIO
F	3.5	0	138.2	137.8	21.1	116.7	0.36	15.3	84.6				382.7: 1
M	5.0	0	577.9	575.8	73.4	502.4	2.06	12.7	87.2				279.5: 1
F	6.5	0	1310.2	1296.6	488.4	808.2	13.6	37.6	62.2				95.3: 1
F	7.5	30D	849.1	826.1	422.5	403.6	23.0	51.1	48.8				35.9: 1
F	10.0	0	3861.5	3843.6	571.0	3272.6	17.9	14.8	85.1				214.7: 1
F	1.W	1W	2731.9	2713.7	502.3	2211.4	18.2	18.5	81.4				149.1: 1
M	2.W	2W	1128.8	1108.6	440.2	668.4	20.2	39.7	60.2				54.8: 1
M	5.W	5W	1077.8	1052.6	505.6	547.0	25.2	48.0	51.9				41.7: 1
M	3.M	3M	1339.1	1289.5	946.5	343.0	49.6	73.4	26.5				25.9: 1
M	1.Y	1Y	1401.6	1315.1	1105.1	210.0	86.5	84.0	15.9				15.2: 1
F	3.Y	3Y	1512.2	1330.9	1330.9	000.0	181.3			261.1	984.9	84.0	7.3: 1
F	13.Y	13Y	2731.0	2508.2	2508.2	000.0	222.8			412.5	1826.0	269.0	11.2: 1
F	15.Y	15Y	3429.8	3185.0	3185.0	000.0	244.8			714.7	2308.6	160.7	13.0: 1
F	17.Y	17Y	3822.0	3526.5	3526.5	000.0	295.5			641.9	2606.4	275.1	11.9: 1



GROWTH OF THE NEGRO SUPRARENAL GLAND

Figure 1

that the weights of the glands increased markedly as fetal length approached 50 cm. At birth the total volume of the gland was 3861.5 cu.mm. The medulla formed 17.9 cu.mm. and the fetal cortex 85.1% of the total cortex. During the latter part of the prenatal period the fetal cortex became increasingly hyperemic, and at birth the center of the fetal cortex often showed beginning evidence of nuclear and cytoplasmic degeneration.

The cytological details of the postnatal degeneration of the fetal cortex have been very well described by Benner ('40) and her description has been confirmed, essentially, by our observations. At the end of the first postnatal week the total volume of the gland was reduced to 2731.9 cu.mm., and by the end of the second postnatal week, to 1128.8 cu.mm. During the first 2 postnatal weeks the adult cortex grew rapidly and comprised 39.7% of the total cortex at the fourteenth day. As a result of the loss of fetal cortex the cortico-medullary ratio was reduced to 54.8:1.

During the period of postnatal degeneration the stroma of the fetal cortex became increasingly evident as the cells disappeared. This stroma was very prominent at the end of the second postnatal week and gradually became more compact as the medulla expanded from within and the adult cortex grew from the outside. The connective tissue stroma of the fetal cortex formed the "capsule" to which Kern ('11) referred. Lewis and Pappenheimer's ('16) observation that there is no new formation of connective tissue is substantiated by this study. However, their report that no growth of the true cortex occurred before the third postnatal year is not confirmed by these data. At the end of the third month the adult cortex formed 73.4% of the total cortex and the stroma of the fetal cortex occupied 26.5% of the total cortex.

At the end of the first year the compact band of connective tissue, which represented 15.9% of the total cortex, remained as a vestige of the stroma of the fetal cortex. During the second year the band of connective tissue became obliterated as the medulla and adult cortex increased in volume.

Throughout childhood both the medulla and cortex grew rapidly and the cortico-medullary ratio correspondingly decreased. During late childhood and near puberty there was an acceleration of cortical growth. Although the glands studied volumetrically do not enable one to draw conclusions regarding the first appearance of the sexual differences which have been found in the adult (Swinyard, '40), it appears that the sex differences may begin at the time of puberty when the adult cortex grows at an accelerated rate.

These data do not support Goldzeiher's opinion that the glands attain morphologic stability in the eighth year. There was considerable variation in the cortico-medullary ratio and total volume throughout childhood and adolescence. In most glands the adult cortico-medullary ratio was reached during the third year of postnatal life. The minimum adult volume was occasionally reached at puberty, however, the average adult volume was not generally equaled until the seventeenth year.

The adult cortex of the newborn is primarily composed of nests of cells arranged in whorls and represents the zona glomerulosa of the adult gland. The vertical alignment of cells which represents the beginning zona fasciculata first appeared during the second postnatal week. The zona reticularis made its initial appearance during the third postnatal month, and its formation appeared to be related to the mechanical forces of growth as the medulla increased from within and the adult cortex expanded inwardly.

In a specimen whose crown rump dimensions indicated a fetal age of $7\frac{1}{2}$ lunar months but which survived for 30 days, involution proceeded normally (table 1). The gland was comparable volumetrically to that of the 30-day infant which was born at full term. This substantiates Lewis and Pappenheimer's report that the involution is not affected by prematurity. Involution was also observed to occur in cortical nodules which contained no medulla and which were located outside the capsule of the gland.

SUMMARY

1. Accurate volumetric determinations of the fetal cortex, adult cortex and medulla of negro suprarenal glands from individuals ranging from $3\frac{1}{2}$ lunar months to 17 years of age were made.

2. At the end of the first trimester of prenatal life the medulla was found to be scattered in small clumps of cells and constituted less than 1.0% of the total gland volume.

3. The medulla grew slowly and steadily throughout the fetal period, infancy and childhood.

4. The fetal cortex grew very rapidly during the last trimester of prenatal life and at birth constituted 85.1% of the cortex. The degeneration of the fetal cortex during the first 2 weeks of postnatal life resulted in a loss of 50% in total gland volume.

5. The stroma of the degenerating fetal cortex occupied 15.9% of the total gland at the end of the first year and practically disappeared by the end of the second year.

6. The adult cortex grew at an accelerated rate during the second and third trimester of prenatal life and again during late childhood and puberty. It comprised 14.8% of the total cortex in the newborn and doubled its volume within 3 postnatal months.

7. The adult cortico-medullary ratio was reached in the third year, but the total gland volume did not reach adult size until the seventeenth year.

8. The adult cortex of the newborn is entirely zona glomerulosa. The zona fasciculata first appeared during the second postnatal week. The zona reticularis made its initial appearance during the third postnatal month, and its formation appeared to be related to the mechanical forces of growth.

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TRABECULAE TRAVERSING HUMAN BURSÆ

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TWO PLATES (SEVENTEEN FIGURES)

In the course of routine dissection in the regular class work in anatomy, discrete strands of tissue were observed to pass through some of the bursae, especially one on the plantar aspect of the foot. Finding no adequate account of such strands, or trabeculae, it seemed desirable to make a more careful examination of the bursae that could be studied in the laboratory. Seventy-nine bodies were wholly or partly dissected while this study was being made and through the cooperation of student dissectors it was possible to see a rather large number of bursae.

Most of the bursae examined were essentially "normal" although many of them showed tabs or appendices of fatty or fibrous tissue extending into the lumina, and not infrequently multilocular bursae were encountered. It was generally difficult to determine whether the multilocular bursae had originally developed in this form or had resulted from the partial disappearance of tissues separating two or more bursae that had arisen independently. Bursae presenting this problem were particularly common in the pre-patellar region. It is not the purpose to enter into a discussion of these multilocular bursae but rather to call attention to the diversity of tissues that are found as free strands crossing the lumina of existing bursae and to note some of the alterations in these tissues.

It is to be presumed that some of these strands were originally outside the bursae and subsequently became in-

cluded through investment by the bursal "membrane" and that others are remnants of originally intervening walls between coalescence bursae. The differences between these two possible modes of formation may not be very great.

TISSUES COMPOSING THE TRABECULAE

As will become clear below, the composition of a trabecula is determined by the locus of its bursa. If the bursa develops in such a way as to include a region of skeletal muscle, the strands may be made up of muscular fasciculi; if in the region of blood vessels, they may include arteries or veins; if in the region of nerves, they may even include nerve bundles. After study by gross methods, representative trabeculae were subjected to the usual histological procedures and studied microscopically.

Collagenous connective tissue (figs. 1-6). Gross dissection shows connective tissue trabeculae of two forms: sheet-like and cord-like. Both types are visible in the pre-tibial bursa shown in figure 1. They are likewise common in the olecranon bursa, and may occur in other constantly occurring or anomalous bursae.

Sheet-like trabeculae such as can be seen in figure 1, appear to arise as remnants of the connective-tissue wall between originally superimposed bursae. They are of the order of 1 mm. in thickness and may taper in width from a few centimeters down to fine strands.

The strand trabeculae may be simply dense collagenous connective tissue as seen in the cross section of the trabecula shown in figure 2, or may be diffusely infiltrated with fat cells as in the cross section of the trabecula of figures 3 and 6.

If the strand is of a diameter greater than 1 mm., its periphery is apt to be capsule-like as seen in figures 2 and 3 (cross sections of strands, 1 x 2 and 1 x 3 mm., respectively); if on the other hand, the strand is of a diameter less than 1 mm., the capsule is apt to be reduced or lacking as is the case with the small strand shown in figure 4 and the finer strands of figure 5.

Connective tissue strands of special origin (figs. 5, 6). Not all of the trabeculae of the bursa shown in figure 1 lend themselves readily to the interpretation of origin by partial destruction of an inter-bursal wall. Strands "D" and "E" of figure 1, although largely connective tissue, are not mere remnants of inter-bursal wall, but are traced as projections of the pre-patellar plexus of nerves.

Cross sections of two of the smaller cords whose composition is now largely connective tissue but which probably arose by a gradual dissection of a once functional structure from the bursa wall, are shown in figures 5 and 6. The central structure of figure 5 was probably a small blood vessel, although it is now so atrophic as to leave some doubt as to this interpretation. Similarly, the small, central, atrophied structure of figure 6 is tentatively identified as a small nerve bundle. Whatever their original structure, even their central structures are now essentially connective tissue.

Adipose connective tissue. Besides the fatty component of the strands mentioned above, such as in figures 3 and 6, there are occasional bursae in which tabs of adipose tissue project into the spaces between trabeculae. The origin of these tabs is not clear. They measure up to a few mm. in diameter, are lipoma-like in gross appearance, are composed of large fat cells, and have a thin but well defined capsular covering of connective tissue.

Bone (fig. 7). Since anomalous calcification and bone formation under various conditions is not unusual, it is perhaps not surprising to find ossification in relation to these trabeculae. One case was found in an enlarged trochanteric bursa which perhaps had been inflamed. The ossified portion of the trabecula made up a terminal knob, a flattened ovoid mass of almost 1 cm. in its greatest diameter, on a peduncle of dense connective tissue. There were several smaller strands, apparently similar to the peduncle, extending entirely through the bursa.

The bony structure is surrounded by a 1-mm. zone of dense irregularly arranged connective tissue. This grades into a

thinner, irregular zone of callus-like bone, within which is a sharply but irregularly limited zone of compact bone of about 7×2 mm. cross section. Not only are Haversian systems with their central canals, concentric lamellae and lacunae to be seen, but even interstitial lamellae. Circumferential lamellae external to this typical compact bone are lacking; instead there is the layer of callus mentioned above.

Skeletal muscle (figs. 8, 9). Trabeculae consisting of skeletal muscle fasciculi were found extending through two bursae below the ischial tuberosity. The more superficial bursa involved the gluteus maximus and the deeper involved the head of the semitendinosus muscle. The muscle fasciculi were separated one from the other and from the bursal walls by dissection of the bursal space within connective tissue layers; i.e., by separations within the perimysium and within the epimysium. Some of the fasciculi were converted to connective tissue strands over a good part of their length; others were apparently still functional skeletal muscle throughout. Strands from the coarse-grained gluteus maximus were mostly of 1- to 3-mm. diameter, but reached widths of as much as 7 mm. A cross section of one of the trabeculae from the semitendinosus muscle is shown in figure 8 and a longitudinal section from the same trabecula in figure 9. A good vascular supply and striations typical of skeletal muscle are clearly visible.

Nerve-artery trabeculae (figs. 10, 16, 17, table 1). The deep, fascial bursae (not the occasional bursa within the plantar fat pad) on the plantar aspect of the fifth metatarso-phalangeal joint had compound nerve-artery trabeculae in about half of the cases in which bursae were found; the data from those bodies dissected in 1942 on which the bursae of both feet could be studied, twenty-five in all, are collected in table 1, and can be summarized as follows:

The bursae were found in 40% of the bodies and the trabeculae in 20%; less than half of these, i.e., less than 10% of all the bodies showed the trabeculae extending as completely free strands across the bursae. The incidence of bursae and of trabeculae tended to be symmetrical with slightly greater

development on the right side; they were perhaps more frequent on feet with some toes removed and on one legged bodies. As seen in table 1, the incidence of comparable bursæ and trabeculae at the first metatarso-phalangeal joint is low; it is rare in relation to the other digits.

Figure 16 (retouched outside the bursa to bring the nerve and artery into relief) shows a trabecula that is free from the wall as it traverses the cavity. Figure 17 (not retouched, but with the nerve, artery and trabecula colored in the dissection before the body was photographed) shows a trabecula which is attached throughout its length to the lateral bursal wall by a thin sheet of connective tissue. In one case, only,

TABLE 1

Incidence of compound nerve-artery trabeculae in the bursæ on the plantar aspect of the metatarso-phalangeal joints. Data from 50 feet (twenty-five bodies).

SIDE AND LOCATION	NUMBER HAVING BURSÆ	NUMBER WITH TRABECULÆ		
		Separation		Total
		Partial	Complete	
First metatarso-phalangeal joint				
Right	11	1	0	1
Left	5	0	0	0
Fifth metatarso-phalangeal joint				
Right	11	4	2	6
Left	10	4	1	5

did the plantar nerve and artery to the fifth digit traverse the bursa as separate trabeculae. In the typical case, the nerve and artery made up a compound trabecula as shown in figures 10, 16, 17. The structures of the nerve and artery are described below.

Arteries (figs. 10, 11, 16, 17). The artery of the trabecula traversing the bursa of the plantar aspect of the fifth metatarso-phalangeal joint is indicated in the cross section of figure 10 by letter "A" and is shown enlarged in figure 11. At this level, i.e., within the bursa, the lumen is completely obliterated and the walls much reduced. The extensive changes in the wall and the apparent obliteration of the lumen

would make the artery almost unrecognizable and might lead to confusion with the artery supplying the nerve bundle were it not for its continuity proximally and distally with the artery supplying the lateral part of the fifth digit. This is in contrast with the hypertrophy of the portions just proximal and distal to the bursa, the wall alone being as thick there as this entire remnant within the bursa (fig. 11); proximally and distally it is especially the intima that is hypertrophied, but not to the extent of closing the lumen. In the region, within the bursal wall, where there is the maximum connective tissue development around the vessel, the lumen is for the most part obliterated by a combination of hypertrophy and contraction of the vessel wall.

Veins. Although it would seem probable that veins should form the basis of trabecular structures just as do arteries, their more superficial position places most of the larger ones beyond the outer walls of most bursae. With smaller vessels, the distinction between veins and arteries in microscopic preparations is made doubtful because of modifications of the vessel walls within the trabeculae; hence although many of the smaller vessels seen in sections probably were veins, their identification is not certain. Only one vein serving as the basis of a trabecula was found. This was a minor branch of the great saphenous (anomalously enlarged with a lumen over 1 mm. in diameter) traversing a pre-tibial bursa just below the knee joint. This trabecular vein was patent as could be seen by exerting intermittent pressure which caused small bubbles to move back and forth within the lumen. Small functional veins incidental to the vascular supply of trabeculae are, of course, much in evidence (cf. fig. 6).

Capillaries (fig. 9). As might be expected, capillaries are readily visible in many of the sections. One is indicated in longitudinal section in figure 9.

Nerves (figs. 1, 12-17). Figure 1 shows branch trabeculae, "D" and "E", which were traced back to the saphenous nerve, and figures 16 and 17 show trabeculae of which the nerve component can be traced proximally to the lateral

plantar nerve and distally to the distribution of the lateral border of the fifth digit. As can be seen even in these photographs of gross dissections, there is a marked hypertrophy of the connective tissue components.

The histological interpretation would be far less certain if it were not for the possibility of comparison with a nerve from a similar region but not involved in a bursa. Figures 12-15 are presented for this purpose, figures 12 and 13 being enlargements of a nerve bundle from the trabecula shown in figure 10, figures 14 and 15 being enlargements of a normal nerve from a similar region not in a bursa (a branch of the medial plantar nerve at the level of the second metatarsophalangeal joint).

It is at once clear that both of these nerves are different from those most often seen in sections and figured in text books. The difference lies in the excessive development of the connective tissue components, including an increase in endoneurium as well as perineurium and epineurium. The extent of the connective tissue development appears to be a function of the exposure to mechanical stress, the development being greatest in these plantar nerves which are subjected to greatest pressure from the weight of the body, intermediate in the superficial nerves of the arm (some of which were examined in this connection), and relatively little in the deeply buried sciatic nerve.

The staining properties of nervous tissues from these dissecting room bodies, while not all that might be desired, are adequate for ready identification of individual fibers in nerve bundles that are devoid of collagenous connective tissue as is shown by the clear figures of Corbin and Gardner ('37). They examined spinal roots from within the spinal canal. The staining qualities of at least the larger nerve fibers of the plantar nerves, whether normal or in bursal trabeculae, are likewise adequate for identification, as can be seen in figures 13 and 15, it being only necessary to recognize the intervening tissue as endoneurium.

The hypertrophy of connective tissue elements of these plantar nerves that pass through bursae as trabeculae, is not limited to any one layer. Thus, from figures 12 and 14, which compare equal magnifications of normal and bursal nerve bundles with roughly similar cross sectional areas, it will be seen that the perineurium of the trabecular nerve is more than twice the normal thickness. Similarly in figures 13 and 15 it will be apparent that the endoneurium of the trabecular nerve greatly exceeds that of the normal nerve. Even the epineurium is much hypertrophied as seen in figure 10. As a result of the obscuring effect of the dense connective tissue, only the larger myelin sheathed axones can be easily identified in these preparations. Presumably they are functional.

Besides the nerve-artery trabeculae associated with the fifth and first digits, there was one case (fifth digit) in which the nerve traversed the bursa as a trabecula by itself separate from that of the artery. There were also occasional cases in which a minor nerve branch traversed this bursa, and one similar case in an uncommon plantar bursa at the second metatarso-phalangeal joint.

Other nerve trabeculae with excessive connective tissue development occurred not only in the pre-patellar bursa of figure 1, but also in pre-tibial bursae, one outstanding case being that of a leg with an amputation 10-15 cm. below the knee joint. Thickening of the nerves in this instance was apparently due to increased pressure caused by an artificial limb, the bursa was highly developed and branches of the infrapatellar plexus occurred as trabeculae.

All of the trabecular nerves studied were of largely cutaneous distribution; none, with deep distribution were encountered.

DISCUSSION

Pressure on trabeculae. Since bursae are subjected to unilateral pressure at right angles to the plane of the bursa, it follows that the trabeculae would also be subjected to unidirectional pressure and this, perpendicular to the lengths of the strands. The unidirectional nature of the pressure would

lead to special strains on the strands which would not be expected were the pressure equal in all directions, as it tends to be, e.g., when the cavities are distended with fluid. This unidirectional pressure may be directly or indirectly responsible for some of the changes observed; e.g., the high development of the connective tissue components of nerve bundles. Under severe working conditions the unidirectional nature of the pressure may be modified to more nearly the conditions of equal pressure in all directions by accumulation of fluid within the bursae, but these conditions are the exception rather than the rule.

Bursal membrane. A bursal membrane, like a synovial membrane of a joint cavity, is not a true membrane, for far from being covered by epithelium, its surface is even made up in part of non-cellular components, the fibers of the connective tissue. The bursal membrane has even fewer connective tissue cells on its surface than does the synovial membrane. This is perhaps in line with the unidirectional nature of the pressure to which it is subjected. The membrane is nevertheless different than a mere surface of a connective tissue cleft for the opposite walls of the bursa do not ordinarily form adhesions, and those bursae arising in the fetus, at least, arise from a peculiarly arranged tissue which cleaves easily in layers (Whittaker, '10).

If the trabeculae are thought of as surrounded by the bursal membrane, they may be considered to be outside the bursa proper. This would be the case especially if the trabeculae develop by dissection from the bursal wall with investment by the membrane. Just how far this would apply in the case of trabeculae formed by wearing away of an interbursal wall is not clear; however, the final result is much the same, for the surface of even these strands can not be distinguished from the adjacent bursal membrane; the similarity is as great physiologically as histologically for the trabeculae remain as discrete strands rather than fusing, even though they abut upon one another.

Composition of trabeculae. Although the trabeculae may be of almost any tissue, some tissues are more common than others. Bursae most commonly form within or between fascial layers; thus, the bursae lie within connective tissue; incidentally, connective tissue strands are the most frequent trabeculae. Similarly, when anomalous bursae form in subcutaneous tissues rather than in fascial layers, as e.g., in the plantar pads of fatty subcutaneous tissues, tough irregular connective tissue strands are apt to make up the trabeculae.

Arteries and nerve bundles, lying within the fascia, form perhaps the next most frequently encountered trabeculae; however, for the most part they pass around bony eminences where bursae are apt to form and are not common as trabeculae. Consistent with the position of the larger superficial veins in the subcutaneous layers rather than in the fascia, and with their courses around bony prominences, no large veins other than one pathologically enlarged one, were found as trabeculae. Muscular fasciculi as trabeculae are also rare and then appear to arise by extension of bursae from fascia into muscular regions.

Changes in trabeculae. Changes in the tissues of the trabeculae may be either atrophic or hypertrophic. Some of the trabeculae apparently undergo fragmentation and destruction by attrition in a manner comparable to the "use destruction" occurring in joints and bursae as described by Meyer ('24, '37; cf. also Codman, '34).

For trabeculae of specialized tissues, there is commonly hypertrophy of the connective tissue elements even though the specialized tissue itself may undergo atrophy. In the case of nerves and blood vessels, the connective tissue development may serve for a time, at least, to protect the functional elements from mechanical distortion and even in the case of atrophic muscle trabeculae the connective tissue development may facilitate functioning of the remaining intact ends of the fasciculi. When the atrophy has become complete in the case of nerves, or the functional state lost as in the occlusion of

a blood vessel, the reinforced remnant may persist as a "connective tissue strand".

SUMMARY

Strands occurring as trabeculae in human bursae probably arise in two different ways, through fenestration of the wall between superimposed bursae and through gradual investment by the bursal "membrane" to the end that structures adjacent to the bursae finally become included as trabeculae.

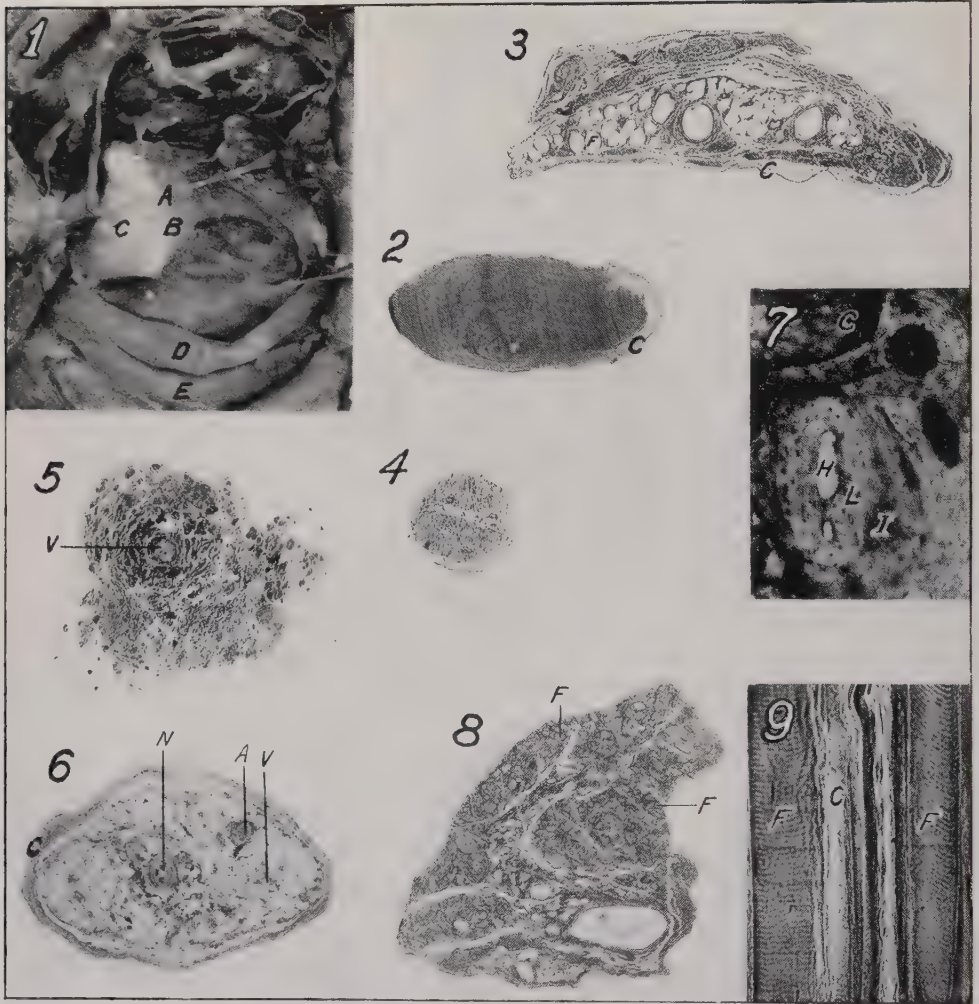
Both from gross dissection and from microscopic examination, it was found that the trabeculae may be of variable composition, the tissues being those of the regions of the bursae. Tissues and structures found were: collagenous connective, adipose, compact bone, skeletal muscle fasciculi, arteries, veins and nerves.

In the trabeculae of specialized structures, there is commonly hypertrophy of the supporting components and often atrophy of the essential elements. In the event of such atrophy of the specialized tissue, the whole structure may be converted secondarily into what would appear to be a simple connective tissue trabecula.

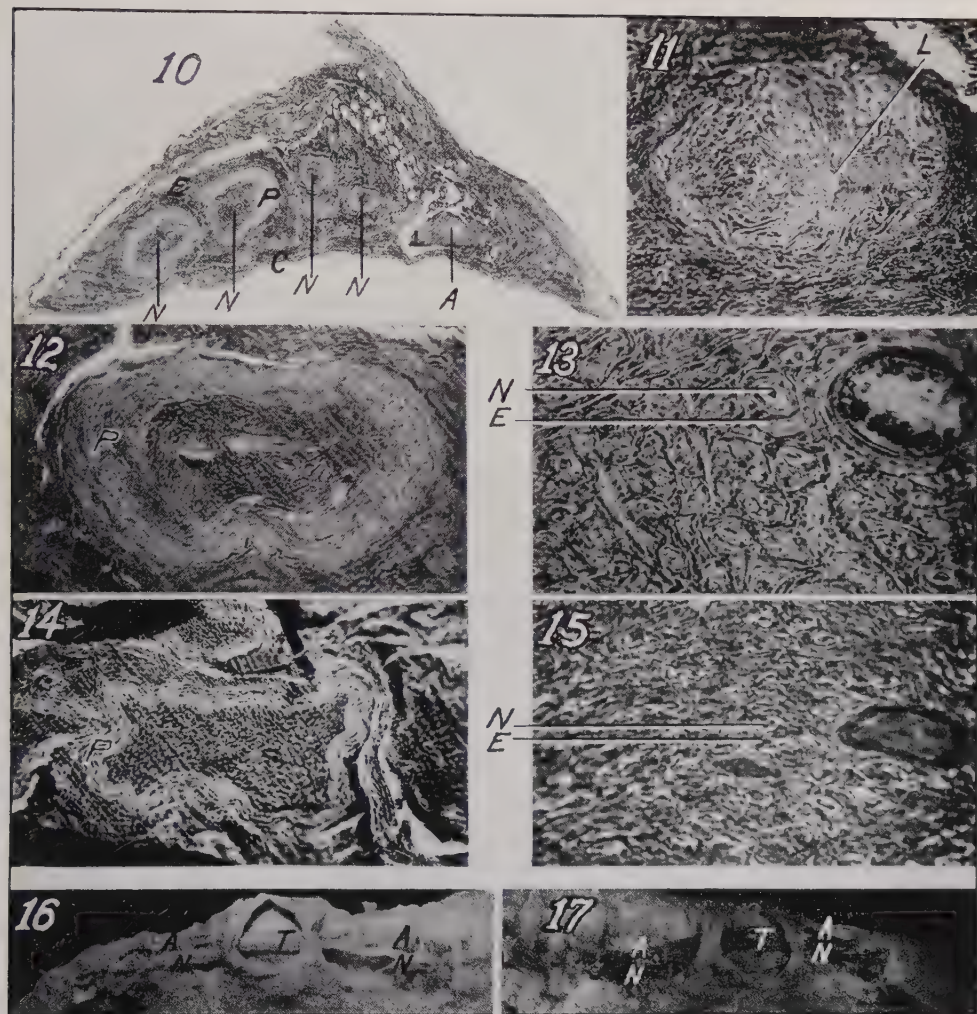
The author wishes to express appreciation to the Anatomy Department, and especially to George Scott, to Prof. H. Kirkman and to Prof. C. H. Danforth for advice and criticism and for making this investigation possible.

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- 1 Prepatellar bursa. A, Cord trabecula; B, sheet trabecula; C, cotton placed in deep bursa; D and E, nerves. 1 $\times$.
- 2 Dense connective tissue trabecula. C, Capsule. 15 $\times$.
- 3 Fatty trabecula. F, fat cell; C, capsule. 15 $\times$.
- 4 Dense connective tissue trabecula. No capsule. 15 $\times$.
- 5 Dense connective tissue trabecula. V, probable central vessel, (atrophied). No capsule. 15 $\times$.
- 6 Fatty trabecula. N, probable central nerve (atrophied); A, artery; V, vein; C, capsule. 15 $\times$.
- 7 Bone. H, Haversian canal L, Haversian lamellae; I, interstitial lamellae; C, callus. 30 $\times$.
- 8 Skeletal muscle, cross section. F, muscle fiber. 15 $\times$.
- 9 Skeletal muscle, longitudinal section. C, capillary with r.b.e.; F, striated muscle fiber. 90 $\times$.



- 10 Plantar bursal trabecula, cross section. A, artery (atrophied); N, nerve bundle; P, perineurium; E, epineurium; C, capsule. 15 X.
- 11 Degenerated artery of fig. 10. Note wavy elastic fibers. L, position of obliterated lumen. 200 X.
- 12 Nerve bundle of trabecula shown in fig. 10. Compare perineurium, P, with perineurium of normal nerve of fig. 14. 60 X.
- 13 Enlargement of nerve from trabecula of fig. 10. N, nerve axone; E, endoneurium. Compare with normal nerve of fig. 15. 300 X.
- 14 Nerve bundle of normal plantar nerve. Compare perineurium, P, with trabecular nerve of fig. 12. 60 X.
- 15 Enlargement of normal nerve of fig. 14. N, nerve axone; E, endoneurium. Compare with trabecular nerve of fig. 13. 30 X.
- 16, 17 Plantar bursae at the fifth metatarso-phalangeal joint and their trabeculae. A, artery; N, nerve; T, trabecula in bursa. 1 X.

EFFECTS OF BLASTEMA TRANSPLANTATIONS ON REGENERATION PROCESSES OF LIMBS IN AMBLYSTOMA LARVAE

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EIGHT FIGURES

INTRODUCTION

Studies of regeneration in both Urodela (Butler, '33; Puckett, '36; Thornton, '38a and '38b) and in Anura (Schotté and Harland, '43a) have clearly shown that amputation of a limb elicits profound changes in the tissues at or beneath the amputation surfaces. There is first an important macroscopically invisible phase, the phase of dedifferentiation, consisting essentially of a loss of histological characteristics of all the formed elements of the limb. With the appearance of the blastema, composed mostly of dedifferentiated elements from the amputation area, the macroscopically visible phase of regeneration begins, characterized by growth and differentiation of new structures.

On the strength of convincing experimental evidence, Butler and Puckett ('40) have suggested that in some way, the appearance of a blastema causes the dedifferentiation processes to subside. Conversely then, the absence of a blastema, should result in disturbances in the cellular equilibrium between dedifferentiation and regeneration proper. Such a condition was obtained in experiments on urodele larvae (Schotté and Butler, '41; Butler and Schotté, '41) and on anuran tadpoles (Schotté and Harland, '42 and '43b), where macroscopical and histological observations concurred to show that no blastema ever forms in nerveless amputated amphibian

limbs. Consequently, if after amputation, limbs were maintained nerveless for any length of time, progressive dedifferentiation took place resulting in resorption of entire limb segments and, in some extreme cases, even of whole limbs. Related to these facts is the interesting discovery of Thornton ('43) that an agent which stops proliferation, such as colchicine, is also able to prevent blastema formation, the result being continuous dedifferentiation with subsequent resorptions.

After these histological studies had enabled the above authors to correlate the normal sequence of the early phases of regeneration with the presence of a blastema, an experimental demonstration of such a correlation was provided by the transplantation of young blastemas on denervated amputated limbs of larval Urodela (Schotté, Butler and Hood, '41). In these experiments the authors were able to show that a young blastema transplanted on a freshly amputated and denervated limb stump actually prevented the mentioned extreme dedifferentiations.

However, the positive outcome of the above experiment could offer only suggestive evidence for an interpretation of the mechanism of action of a transplanted blastema. For all we know, the transplanted blastema might produce the same effect in many different ways, possibly totally unrelated to the dedifferentiation phase of regeneration. In the absence of adequate histological confirmation it was not possible to determine whether the blastema had merely slowed down the dedifferentiation processes or suppressed them altogether. Furthermore, denervated limbs representing, as it were pathological conditions differing extremely from those found in normal limbs, might not have been too happy a choice for the experimental investigation of the role of the blastema as a controlling agent of the early dedifferentiation processes.

If Butler's interpretation of the role of the blastema as an agent regulating the extent of dedifferentiation in normal regeneration reflects actual processes, then it was to be expected that the transplantation of a young blastema onto a

normal limb immediately after amputation should so interfere with the early phases of regeneration as to cause the suppression of the dedifferentiation phase altogether. For that reason, the method chosen to test anew Butler's hypothesis was the autoplasmic or homoplasmic transplantation of an early limb blastema on freshly amputated limbs combined with a systematic histological investigation of its effects on the tissues of the stump.

MATERIALS AND METHODS

The material used for this research consisted chiefly of young *Amblystoma* larvae collected from ponds in the vicinity of Amherst, and of a few larvae of *A. opacum* raised from eggs in the laboratory. At the time of operation, these larvae varied in length from 18 to 55 mm., the majority of the experimental specimens measured, however, from 25 to 35 mm.

Blastemas were secured either from another limb of the host itself, or from limbs of a different donor: in autoplasmic transplantations blastemas were secured from the zeugopode region of a previously amputated right limb; in homoplasmic transplantations, either a fore or a hind limb amputated several days previously served as blastema donor. The age of the transplanted blastemas varied from 5 to 9 days.

Operative technique. Both larvae, the donor with its blastema and the host, under narcosis in M.S. 1:5000, are placed next to each other in a 15-cm. petri dish, on gauze moistened with a mixture of M.S. solution and saline. All the operations are, of course, performed under the dissecting microscope. The left arm of the host animal, together with the right control arm, are then amputated through the stylopode at the same rather distal level. The blastema transplant is rendered more visible by staining it slightly in a Nile Blue Sulfate solution about 5 minutes before the transplantation. We have found that this superficial stain with Nile Blue is sufficiently strong to enable the distinction of the transplant from the host tissue for the duration of the experiment (not longer than 9 days).

The blastema is removed from the donor limb with iridec-tomy scissors, and placed on top of the amputated stump with the aid of watchmaker forceps. In order to maintain the transplant in position, so as to allow its healing to the fresh amputation surface, the left upper arm is kept for several hours in an upright position with the aid of small pieces of silk thread serving as supports. It is advisable, at this stage, to expose limb and transplant to the warmth of the operation lamp until limb and blastema shrink slightly through drying. After this the animal, still narcotized in half strength M.S. solution, is kept for at least 24 and better 48 hours, in the refrigerator at about 10°C. The larvae are then released and examined under a dissecting microscope for the presence of the transplant. The operation is successful in about 40% of the cases.

Fixation for histological study was done between 3 and 9 days after the transplantation of the blastema and the amputation of the right control limb. Sections were made at 7 μ , and always stained in Harris' Hematoxylin, with Orange G as counterstain.

EXPERIMENTAL RESULTS

In an experiment in which the action of a transplanted blastema on the tissues of the amputation surface is being investigated, it is of first importance to determine the nature and the intimacy of the contact ultimately effected between the transplant and its supporting stump. In that respect several possibilities can arise: (1) the blastema covers the amputation surface only partially; (2) the blastema, originally well placed, has subsequently become displaced; (3) the blastema becomes resorbed after a short time; (4) the blastema, although fitting the amputation surface perfectly at first, does not succeed in taking root and is eventually displaced by a new blastema; (5) the blastema fits perfectly, remains in that position for the duration of the experiment and does not become resorbed.

Roughly, all these possibilities can be included in two categories—the partially successful, (points 1–4) and the wholly successful transplantation experiments (point 5).

I. Effects of blastemas covering the amputation surface partially or only temporarily

There were nineteen cases in which the above poor fits or ultimate resorptions of blastemas occurred. From our records and from the original camera lucida drawings, the following examples can be briefly described.

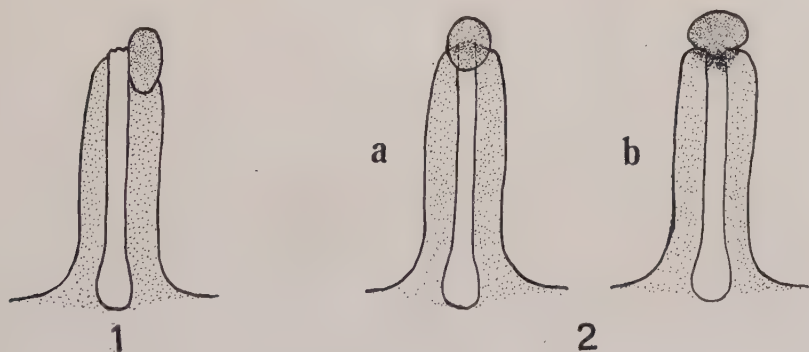


Fig. 1 Camera lucida drawing of left upper arm from a 25-mm. *Amblystoma opacum* larva 3 days after autoplasmic transplantation of an 8-day-old blastema.

Fig. 2a and 2b Camera lucida drawings of left upper arm from a 24-mm. *A. punctatum* larva. a, 3 days after autoplasmic transplantation of 8-day blastema; b, 3 days later, blastema in the process of being walled off.

In case HB 3 represented in figure 1, it will be seen that the blastema is fixed on the dorsal side of the protruding humerus, covering neither the whole of the amputation surface nor the amputated humerus.

In the two successive drawings of case HB 13, another possibility is presented. Here at first (fig. 2a) the transplantation seemed perfectly successful, the whole of the amputation surface being covered by the blastema, and the shaft of the humerus apparently situated in the midst of the blastema tissue. Two days after the operation, however (fig. 2b), the transplant acquired a spherical appearance, and

became eventually constricted at its base by the healing over epidermis of the amputation surface. Although no further modification in the relationship between blastema and amputation stump was observed, it could be seen, on section, that no fusion between the proximal tissues of the blastema and the old tissues of the stump had occurred. Moreover, the blastema epidermis had rounded up into a perfect sphere separating the transplant entirely from the tissues of the stump.



Fig. 3a and 3b Camera lucida drawings of left arm of 22-mm. *A. punctatum* larva. a, 3 days after homoplastic transplantation of 8-day blastema; b, 2 days later.

In the case of HB 16 another possibility can be illustrated. Here, the blastema at first fitted (fig. 3a) perfectly over the amputation surface and over the amputated humeral shaft, but due to the usual contractions, after amputation, of the soft constituents of the limb, the transplant was drawn away into a lateral position, causing the shaft of the humerus to break through the tender epidermis of the blastema (fig. 3b). On section it was found that 7 days after the experiment, the humerus remained still free of epidermal covering.

In case HB 103, the resorption of a well-situated transplant can be observed. Figure 4a shows the usual aspects of a successful transplantation, but 3 days later (fig. 4b) we found an extraordinary regression of the transplant which we attribute to some injury. Eventually, the blastema became

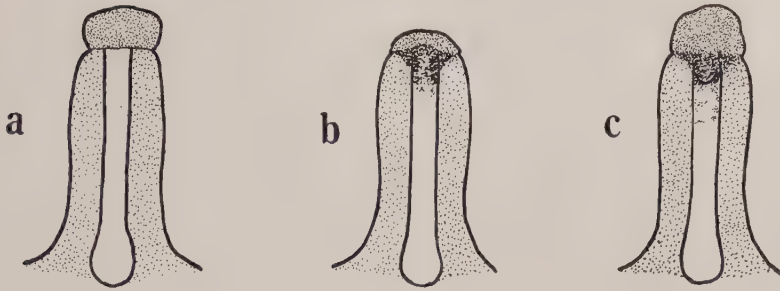


Fig. 4a-4c Camera lucida drawings of 3 stages of autoplasmic transplantation of 7-day blastema on left upper arm of *A. punctatum* larva of 23 mm. a, 2 days after transplantation; b, 2 days later; c, 9 days after transplantation.

difficult to distinguish, and the usual phases of dedifferentiation could be seen through the transparent tissues to occur beneath the receding transplant. As a result, 9 days after transplantation (fig. 4c) a well formed blastema was seen, in no way different from the one which had developed on the simply amputated control limb. There is little doubt that, in this case, an entirely new blastema was formed from the stump and that the transplant vanished totally.

A final example of an only partially successful blastema transplantation was observed in case HB 112. The originally large transplant of figure 5a was seen to retract considerably 4 days later (fig. 5b). From then on, however, the easily visible Nile Blue stained transplant remained unmodified for several days. When 7 days after the transplantation, a last

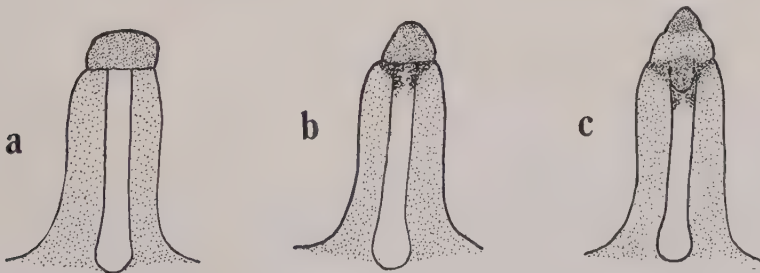


Fig. 5a-5c Camera lucida drawings of 3 stages of homoplastic transplantation of 7-day regenerate on left upper arm of a 31-mm. *A. punctatum* larva. a, 2 days after transplantation; b, 2 days later; c, 7 days after transplantation.

drawing of this case was made (fig. 5c), the still distinguishable transplant was found situated over the tip of a much larger regenerate, which meanwhile had formed, obviously, from the amputation tissues.

The other fourteen cases included in this section all enter into one or the other of the above categories, and they all showed the same effects—the blastema either grew in side-wise or was shifted subsequently into a sidewise position, or else it became partially or completely resorbed. In each instance the stump tissues had formed another, entirely new blastema, except in those rare cases in which the humerus was protruding through the epidermis.

The histological verification of all nineteen cases showed invariably the same picture, namely that the dedifferentiation processes were found to be quite identical in both limbs. In other words, misplaced or readily resorbed transplanted blastemas do not exert any influence whatsoever on the early regeneration phases of the tissues in the amputation area.

II. Effects of successfully transplanted blastemas on dedifferentiation processes of amputated limbs

In twenty cases the transplanted blastemas not only covered the amputation surface entirely, but they also remained attached to the stump without appreciable resorption until the end of the experiment (from 5 to 9 days).

Since most of our larvae were young, many changes within the limb, after amputation and blastema transplantation, could be observed on the living limb as a result of its transparency, as previously described by Schotté and Butler ('41). The differences between the two limbs, discernible particularly in their humeri, were especially striking in the case of HB 79, (autoplastic transplantation of 8-day regenerate on left limb, right limb simply amputated), figures 6a to 6f, (see explanations of figures).

It can be seen clearly from these figures that while in the right limb, without a transplant, the dedifferentiation processes were progressive up to the ninth day, in the left limb,

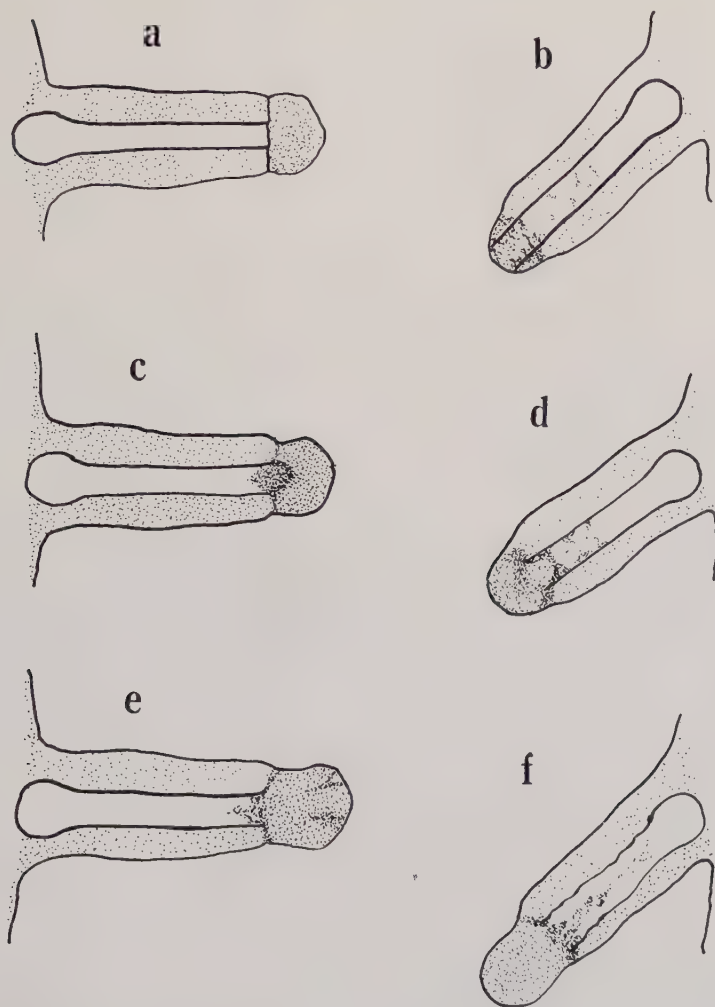


Fig. 6a-6f Camera lucida drawings of left upper arm with transplant (a, c and e) and of amputated right control arm (b, d and f) in an *Amblystoma punctatum* larva of 30 mm. a, left limb 3 days after transplantation; b, right limb 3 days after amputation; the dots at the tip indicate either small blood sinuses or beginning dedifferentiation processes; c, left limb with blastema 5 days after transplantation; d, right control limb 5 days after amputation in the midst of active dedifferentiation processes with beginnings of blastema formation; e, blastema on left limb, 7 days after transplantation shows traces of beginning differentiation; f, right limb 7 days after transplantation showing emptied humeral shaft, wavy perichondral sheath and well-developed blastema.

the amputation surface of which was covered with a blastema, no similar breaking down of the humerus was observed. The comparison between right and left is particularly noticeable 5 and 7 days from the beginning of the experiment. Nine days after amputation, while the humerus of the right limb presents superficially the aspect of a hollow tube, on the left, the shaft of the humerus, protruding into the blastema, shows only the slightest signs of dedifferentiation. The histological investigation confirms the above morphological observations completely. There are only very slight changes in the aspect of the cartilage at the distal tip of the left humerus, while nearly the whole of the perichondral shaft of the control humerus was empty matrix and showed aspects very similar to the ones represented in figure 8 of the next case.

In case HB 104 (*Amblystoma punctatum* larva, 32-mm. transplantation of a 7-day regenerate, fixation of both limbs 8 days after transplantation and amputation), the records of the drawings of both limbs of this case indicate optimal conditions for the experiment—a fairly large blastema placed squarely on top of the amputation surface of the left limb and remaining there for the duration of the experiment; on the control side, the young blastema became apparent at the usual sixth day after amputation. At the time of fixation, the transplanted blastema had elongated a little, but it was ascertained from its still persisting Nile Blue stain that the transplant was not replaced by a new blastema.

A comparison of the sections is instructive. In figure 7 the left limb shows the presence of a still undifferentiated, although quite large, blastema. The extreme distal tip of the humeral shaft is not totally devoid of features occurring in early dedifferentiation processes, but this affects only a small fraction of the humerus. The rest of the cartilage within the perichondral bone is still firmly embedded in its own matrix, no conspicuous vacuolation had occurred, and in all other respects the skeletal structures are rather normal. On the right side (fig. 8), on the contrary, the humerus presents an entirely different aspect. This structure has been practically

emptied of its cartilaginous content, the matrix has been dissolved along its whole length, and the cells visible in the middle of the empty perichondral sheath are free cells which to all intents and purposes have lost the characteristics of cartilaginous cells. The emptying of the humerus is further underlined by the wavy structure of the perichondral sheath, which has obviously lost its inner support. In brief, this

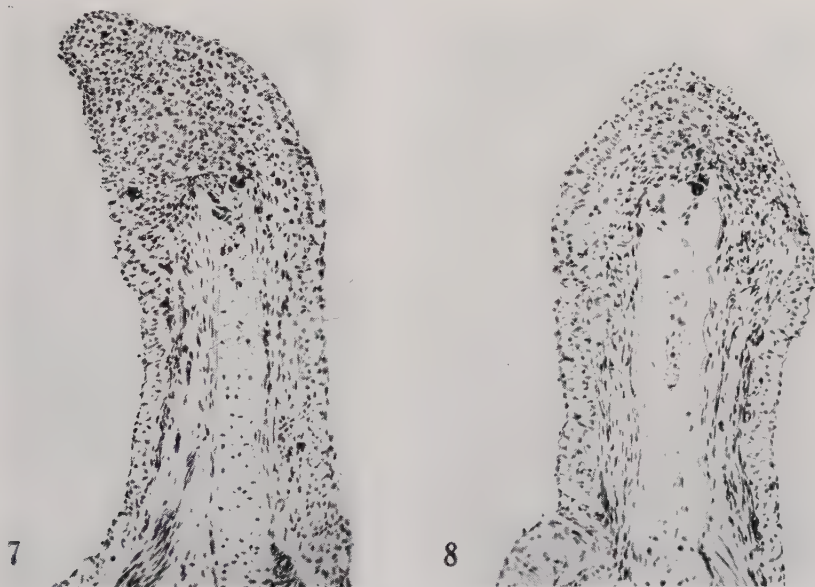


Fig. 7 Longitudinal section of left limb of case HB 104. $\times 60$.

Fig. 8 Longitudinal section of right control limb of case HB 104. $\times 60$.

humerus represents the typical features of a skeletal structure in the midst of dedifferentiation processes, as was so well described by Butler in '33 and again in '41 (Butler and Schotté).

Among the twenty cases there were seven in which the comparison between the limb carrying a well placed blastema and the simply amputated control limb, presented similar very striking differences, in their degrees of dedifferentiation. In seven other cases the comparison between the dedifferen-

tiation phases of the two sides of the animal was suggestive, although not altogether conclusive. Finally, in six cases, no noticeable differences could be observed, on section, in limb stump tissues adjacent to a blastema transplant as compared to those of the control limb.

The careful investigation, from our records, of possible causes which would explain the absence of any modification in the dedifferentiation phases on limbs carrying a blastema transplant was not very satisfactory. In all these cases, it is true, the blastema varied in age, but this was true also of the more successful cases.

There are, however, many factors which might affect the transplants. There is, for instance, no means of ascertaining at what precise moment the transplanted blastema took root on the amputation stump. It is common observation that the amputation surfaces of different individuals present different amounts of hemorrhage, and often one finds on section large blood sinuses which could, in our opinion, interfere successfully with the early fusion of the transplanted blastema. We must consider, also, the possible action of blastemas of different ages, obtained, in addition, from donors of various origins and ages.

Another cause of error, to which not enough attention was paid at the time these operations were performed was discovered while studying the sections. In several cases, pieces of debris of humeral shaft which obviously had been removed from the donor arm were found at the basis of the blastema transplant. While perichondral pieces are easy to detect, it is much more difficult to become aware of possible transplantations of the soft constituents of the limb, a fact which thus multiplies the causes of error. From what we know at present it is unlikely that a blastema, with an appreciable amount of elements from its own base, would affect the dedifferentiation processes of the stump on which it was transplanted. Indeed, Schotté, Butler and Hood ('41) have shown that transplanted older blastemas showing beginnings of histogenetic and morphogenetic differentiations are unable to check dedifferentia-

tion processes of denervated amputated limbs. Moreover, when placed on a stump in the process of active regression, they become involved in the resorption processes of their new base and ultimately disappear themselves.

CONCLUSIONS

From the foregoing experiments it can be seen that while the influence of badly fitting or resorbing blastema transplantations was, in nineteen cases, absolutely nil with respect to the dedifferentiation phases on the neighboring stump, in the twenty cases in which the blastemas were found to fit the amputation surface, pronounced effects were obtained in over two-thirds of the cases. Although these effects were not identical in all the positive cases, in a third of them the differences in the extent of dedifferentiation in limbs with a transplant as compared to a limb without a transplant were so pronounced that no doubt in the mind of the observer was left as to an effective interference with the normal regeneration processes.

It is noteworthy that among the unsuccessful transplants described in the first section, there were many cases in which the blastema at first fitted well over the amputation surface, and was only subsequently displaced. In six other cases of that series, there were excellent fits, but the blastemas became resorbed precociously. In these instances no effects of the transplant were observed. This seems to indicate that the expected action of the blastema cannot manifest itself if merely a transitory contact has been established and that, furthermore, perfect contiguity between cells of the blastema and the tissues of the amputation surface must exist.

Such conditions, detectable in some cases, undetectable or not understood in others, point to the probable explanation of cases described in the second section in which apparently healthy blastemas did yet not exert any appreciable effect on the dedifferentiation processes of stumps on which they were perfectly well placed.

Whether or not the negative cases of these series can be accounted for by these hypothetical causes, there is no doubt that the problem of the role of the blastema as a controlling agent of the early phases of regeneration deserves further attention. New experiments with improved techniques are being undertaken at present in this laboratory to clarify this difficult and vastly important question.

In spite of the above mentioned reservations and even though this research is considered to be only in an advanced stage of preliminary investigation, these experiments strongly suggest that, under ideal conditions, a well placed young blastema is capable of actually suppressing the dedifferentiation phase altogether. Butler's view of the role of the blastema as an agent regulating the cellular interactions of early regeneration thus finds new confirmation.

SUMMARY

The influence of the blastema on the dedifferentiation phase of early regeneration was studied by the method of autoplasic and homoplasic transplantation of young blastemas onto freshly amputated limbs of *Amblystoma* larvae. The blastema was transplanted to the stump of the distal or middle upper arm of the left limb, the right arm, without a transplant, being simultaneously amputated for control purposes. After study of the behavior of the transplanted blastema and systematic observation of the macroscopical changes in both limbs, the larvae were fixed for histological comparison of the two limbs.

The results differed sharply according to the way the transplanted blastemas established themselves on the amputation surfaces of the limb: a) In nineteen cases in which the blastema transplant covered the amputation surface only partially, became ultimately displaced or resorbed, no differences could be detected in the dedifferentiation phases of the two limbs. In other words, misplaced or resorbing blastemas do not influence the dedifferentiation processes at all. b) In the twenty cases in which the blastemas fitted the amputation

surface perfectly and remained there for the duration of the experiment noticeable effects were observed in more than two thirds of the cases; in seven cases, particularly, very conspicuous differences in the status of dedifferentiation of the tissues of the stump were observed on the side carrying a blastema as compared with the simply amputated control limb. In six cases, however, the successful transplantation of a blastema seemed not to affect the extent of the dedifferentiation processes of the amputated limb. Possible causes affecting the results in the negative cases are being discussed.

In spite of the fact that some experimental conditions still escape control, it is concluded that a young blastema transplanted onto a freshly amputated limb is capable, by its presence, of actually suppressing the dedifferentiation phase of regeneration.

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INTRA VITAM STAINING AND TOXICITY OF CHLORAZOL FAST PINK IN MICE AND RATS¹

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TWO TEXT FIGURES AND ONE PLATE (SEVEN FIGURES)

INTRODUCTION

Chlorazol fast pink (Color Index no. 353) is one of the several azo-dyes which are anticoagulants (Rous, Gilding, and Smith, '30; Brambell and Parkes, '32; Huggett, '34; Barratt, '37; Quick, '37 and '42). Subsequent to intravenous injection of this dye the in vitro clotting time of rabbit's blood was prolonged for as much as 12 hours beyond normal (Huggett and Rowe, '33b; Modell, '39). At a dose level of 160 m/kg. chlorazol fast pink prolonged the clotting time for twice as long as did the same amount of heparin (Huggett and Rowe, '33b).

The dye first known as chlorazol fast pink has been used since 1902 for the dyeing of cotton fabrics (Color Index, '24, and Supplement '28) and it has been designated by a variety of commercial names of which approximately seventeen have been listed up to the present time. By far the most frequent designation has been that of "chlorazol fast pink" which has been adopted in this report. Other trade names for the dye have been mentioned by Quick ('37) and Modell ('39). Since

¹ This investigation has been supported by the International Cancer Research Foundation and the Carnegie Foundation of New York.

² Fellow of the International Cancer Research Foundation.

the dye stuff first used was relatively toxic, a method of purification was devised (Modell, '39) and more recently a less toxic product has become commercially available.

Chlorazol fast pink has been utilized as an anticoagulant in laboratory experiments involving the use of cannulae placed in blood vessels (Jackson, '39). The dye was effective when given intravenously before or during the experiment and was also satisfactory when introduced into the pressure system of a recording manometer near the junction of the cannula and tubing (Modell, '39). During the course of similar experiments the writers and others have noted that a generalized intense coloring of the animal occurred following intravenous injection of the dye and that some of the dye was rapidly excreted in the urine.

The following experiments were undertaken to determine (1) the morphological distribution of the vital staining, (2) the minimum lethal dose, and (3) the toxic reactions including the effects on bleeding and coagulation time subsequent to subcutaneous and intraperitoneal injections of this dye.

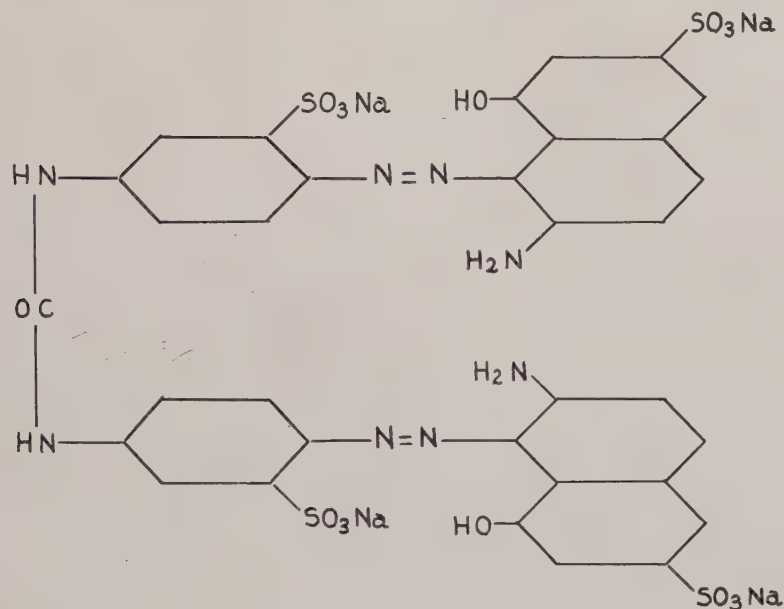
Chlorazol fast pink (fig. 1)³ is a member of the dis-azo group of coloring matters (Conn, '40). Chemically it is related to the well known vital dyes trypan blue (C.I. no. 477), trypan red (C.I. no. 438), and vital red (C.I. no. 456). The dye used in the present study shows positive responses as to the reactions described for the original "Chlorazol Fast Pink BK" listed in the Color Index ('24). These reactions are: (1) a crimson-red solution in water; (2) a reddish-violet solution in hydrochloric acid, (3) a brownish-yellow solution in sodium hydroxide; and (4) a blue solution containing a flocculant blue-black precipitate with sulphuric acid. In addition a light cherry-red solution is formed with ammonium hydroxide, and nitric acid forms a dark black solution containing a finely dispersed black precipitate.

³ The writers wish to thank Mr. N. C. Jacobs for assistance with the illustrative material.

MATERIALS AND METHODS

I. Vital staining

Young adult mice ⁴ of the C3H and C57 strains with an average weight of 22 gm. received twelve daily subcutaneous injections of 0.1 cc. of a 0.5% solution of dye as follows: (1) ten received chlorazol fast pink; (2) five received trypan blue;



$C_{33} H_{22} N_8 O_{15} S_4 Na_4$
Chlorazol Fast Pink(C.I.No.353)

Figure 1

and (3) two received trypan red. Eight of the C3H mice which were treated with chlorazol fast pink had received subcutaneous transplants of a lymphosarcoma (Gardner, Kirschbaum, and Strong, '40) 9 days prior to the beginning of dye injections. The same was true for three of the mice which

⁴The inbred strains of mice used in this study were furnished through the courtesy of Dr. L. C. Strong.

were injected with trypan blue and for both of the mice which received trypan red.

In addition, eight albino rats were injected subcutaneously three times a week for 2 weeks with dye (0.4 cc. of a 0.5% aqueous solution) as follows: (1) four animals with trypan blue; (2) two animals with trypan red; and (3) two animals with chlorazol black E (C.I. no. 581). Studies of vital staining were also made upon some of the rats and mice used for the toxicity and lethal dose experiments. However, to avoid duplication these animals will only be included in the portion of the report concerned with that phase of the experiments.

It was not the purpose of the investigation to study in any detail the vital staining capacity of the several dyes mentioned above. These substances were used so that an adequate number of well known dyes could be compared with the one under particular consideration, chlorazol fast pink.⁵

The histology of the various tissues and organs was studied by means of several methods. The fixatives used were Bouin's, Zenker's acetic acid mixture, Helly's fluid, 10% formalin, Susa's fluid, and a modified Lavdowsky's fluid.⁶ The latter was by far the most satisfactory and the results stated later in the report were subsequent to its use. This fluid is water-clear and requires no washing in water. Also, it facilitates the determination of whether tissues lose any considerable amount of the stored dye material. No dye was observed to be lost during fixation in this solution. Paraffin sections were cut at 10 micra and mounted on glass slides. Subsequent to the removal of paraffin with xylol at least one slide of each tissue was prepared by each of the following methods: (1) mounted in balsam and covered with glass cover slips; (2) stained lightly by the usual method with Ehrlich's hematoxylin with and without eosin; (3) carried through a graded series of alcohols to water and stained rapidly (10 seconds)

⁵ Some of the dyes used were supplied by the National Aniline Division of the Allied Chemical and Dye Corporation.

⁶ Formaldehyde — 10 parts; 95% alcohol — 50 parts; glacial acetic acid — 3.5 parts; distilled water — 40 parts.

in 0.5% aqueous toluidine blue, washed in 95% alcohol, dehydrated in dioxane and xylol, mounted and covered in the usual fashion. Dry imprints were made of spleens, livers, and lymph nodes. The imprints were not fixed or stained and this procedure affords a rapid method for the determination of the presence of a vital dye within cells. Spreads were made of the subcutaneous connective tissue, fixed in the Lavdowsky's fluid, and studied unstained. Blood smears were made on glass slides.

All animals in this series were killed with illuminating gas. In all instances careful notes were made at autopsy as to the gross distribution of the dye.

II. Toxicity and lethal dose

Toxicity studies were carried out on adult albino rats; most of these rats were given solutions intraperitoneally (table 1). However, a small group received intravenous injections. The

TABLE 1
Toxicity of chlorazol fast pink solution (8% aqueous) in rats
(intraperitoneal injection: 115 rats).

A. "Non-toxic" fraction of chlorazol fast pink

NO. RATS	DOSE CC./100 G. RAT	NO. DEAD	% MORTALITY
10	0.1	0	0
15	0.2	3	20
5	0.4	0	0
15	0.6	9	60
15	0.8	12	80
15	1.0	13	87

B. "Non-toxic" fraction of chlorazol fast pink, purified, recrystallized

5	0.4	3	60
5	0.6	4	80
5	0.8	4	80

C. "Toxic" fraction from chlorazol fast pink

15	0.2	0	0
5	0.5	3	60
5	0.75	4	80

LD50 (Probit Kill—5.0) — 0.5 cc. per 100 g. rat

chlorazol fast pink was taken from three samples manufactured by the National Aniline Division. No differences in the behaviour of any of the animals receiving any of these samples were observed. To obtain data on purified and non-purified samples of chlorazol fast pink, the dye stuff as received was treated according to the procedure of Modell ('39). This material is referred to as "non-toxic" fraction. The "toxic" fraction was a combination of the residue from recrystallization plus any material which would not dissolve in the first attempt at solution of the original material. The rats were large adult animals whose body weights ranged from 200 to 450 gm. One hundred and fifteen rats were given intraperitoneal injections. Seven rats were given intravenous injections.

To determine the minimum lethal dose in mice fourteen adult animals weighing approximately 22 gm. each received injections of a 5% aqueous solution of chlorazol fast pink as follows: (1) two mice — 1 cc. intraperitoneally; (2) two mice — 0.5 cc. subcutaneously and intraperitoneally, respectively; (3) four mice — 0.2 cc. intraperitoneally; (4) four mice — 0.2 cc. subcutaneously and intraperitoneally, respectively; and (5) two mice — 0.1 cc. subcutaneously and intraperitoneally, respectively. These mice included six C57 males, four C57 females, and four female Swiss albinos.

III. Coagulation and bleeding time

Bleeding time and in vitro coagulation time were determined in eight Swiss albino mice and two C57 mice which were thereafter treated as follows: (1) four mice received daily subcutaneous injections of 1% aqueous chlorazol fast pink for 6 days; (2) two mice received a single subcutaneous injection of 0.2 cc. of 5% aqueous chlorazol fast pink; and (3) four mice received one injection of 0.2 cc. of 5% aqueous chlorazol fast pink intraperitoneally. In the first group of animals the coagulation and bleeding times were again determined on the seventh day after the beginning of treatment. In the two latter groups coagulation and bleeding times were determined

16 hours after the single injection of dye. The in vitro coagulation times was obtained by the use of a Brodie-Russell-Boggs coagulometer (Hinman and Sladen, '07). Blood was secured by amputating the tip of the tail. Bleeding time was determined at the same site by observation and by very lightly contacting the bleeding site with a piece of filter paper at 30 seconds to 1 minute intervals. The animals were not restrained during the latter procedure but were allowed to remain on a wide-meshed screen which had been placed over a large open container. The tip of the tail usually remained above the wire or in one of the wide meshes.

FINDINGS

I. Vital staining

The vital staining which attended subcutaneous and intraperitoneal injections of chlorazol fast pink was essentially like that which follows the use of the well known dye trypan blue. For this reason no detailed description of the sites of storage and the cells concerned will be given. Of the five dyes used the greatest degree of vital staining was obtained with chlorazol fast pink and trypan blue. Chlorazol black which has recently come into use as a vital stain (Baker, '41) was retained in fair amounts and has some advantages because of its dark color. When chlorazol fast pink was administered at non-toxic levels, storage was limited to the wide spread system of macrophages except in the case of some of the lining cells of the nephron.

The dye caused a generalized coloring of the animal. At autopsy the kidneys and lymph nodes were the most deeply colored organs. Subcutaneous, intraabdominal, and intrathoracic lymph nodes all showed an equivalent degree of staining. The liver and spleen were a darker red than usual. The walls of the gastrointestinal tract were pink and much lighter in color than the uterus. With the exception of the usual lymphoid tissue the thoracic contents showed little gross evidence of dye storage. The central nervous system was not stained.

The dilute basic and acid solutions used in staining the nuclei with Ehrlich's hematoxylin caused a color change in the stored chlorazol fast pink and it appeared as an orange-pink material. Subsequent to staining with toluidine blue the dye inclusions were of a darker red color. When the slides were only passed through xylol prior to mounting in balsam and covering with glass, the vital dye was dark pink in color. The color of the dye has undergone no change in slides made more than 12 months ago.

The structures stained in various tissues and organs are as follows:

Blood. None of the cellular elements contained dye.

Subcutaneous connective tissue. Dye was present as segregated granules in the usual macrophages (histiocytes).

Skin and skeletal muscle. There were a few scattered dye-containing cells in the dermis. In the muscles dye storage was limited to perivascular cells.

Adipose tissue. The peripheral cytoplasmic ring of adult fat cells frequently contained dye. A greater amount was present in the smaller "signet ring" cells particularly in the perinuclear region. The brown fat which is located in rodents in the perirenal area and alongside the abdominal and thoracic aorta was colored by the dye (fig. 3). The small fat cells whose cytoplasm consisted mostly of several fine droplets rather than a single large one contained many intracytoplasmic accumulations of dye. The fat cells of the interscapular fat "gland" did not contain dye. However, numerous dye inclusions were present in macrophages located in the perilobular stroma of this structure.

Lungs. Dye-containing cells were infrequent, but a few were observed in the perivascular stroma.

Heart. Some dye was stored in a small number of cells located in the perivascular connective tissue. The endothelium of the heart and blood vessels was free of dye.

Liver. A majority of the cells lining the sinusoids had stored a large amount of dye in granular form. Some of the largest dye containing cells were free in the sinusoidal channels.

Macrophages in the stroma and capsule showed cytoplasmic inclusions of dye.

Spleen. A relatively few cells showed any evidence of dye storage. A considerable amount of hemosiderin was present in the lining cells of the sinusoids, but little dye. The same was true for certain cells of the reticulum.

Lymph nodes. The lymph nodes were the site of the greatest amount of dye storage. All of the cells lining the intranodal sinusoids demonstrated dye storage (fig. 4). The majority contained granular dye in their cytoplasm. In addition the cytoplasm of these cells contained an amorphous suffusion (non-segregated) of dye, and many dye granules had adhered to or were adsorbed on their sinusoidal surfaces. These three types of dye accumulation occurred to a lesser degree in other sites but were best demonstrated in the lymph nodes. Subcutaneous, intrathoracic, and intraabdominal nodes all showed the same picture.

Gastrointestinal tract. A few macrophages located in the lamina propria and submucosa contained dye. Dye was also present in cells located in the stroma of the pancreas.

Kidney. Dye was observed in the epithelium of the glomerular capsule. The lining cells of the proximal convoluted tubules contained the greatest amount (fig. 5). More distal portions of the secretory tubules were free of dye as were the collecting tubules and ureter.

Adrenal. Dye was limited to macrophages in the capsule, and in the stroma of the glomerulosa and outer fasciculata areas.

Testis. Many of the cells of the interstitium were either stained (cytoplasm) or contained dye in a granular form (fig. 8). The vacuolated and/or granular cells of Leydig seemed only to be stained, that is, to contain dye in a non-granular and non-segregated form. In contrast the other cells seemed to be true macrophages in that definite storage of dye as distinct granules had occurred. However, the distinction was at best difficult. All of the germinal elements were free of dye.

Female reproductive system. The lamina propria and subserosa of the uterus and fallopian tubes contained many dye-filled macrophages. The greatest number was present in the lamina propria of the uterus. Only a few macrophages were observed in the vagina. Dye-containing cells were scattered in the ovarian stroma and many were present in atretic follicles. In the rats and mice numerous dye-containing macrophages were observed in old corpora lutea including those which showed considerable fibrosis.

Nervous system. The central nervous system was free of dye. Dye-containing macrophages were located in the epineurium of peripheral nerves, and in the intraabdominal autonomic ganglia.

Transplanted lymphosarcoma. Many dye-filled macrophages were scattered without any particular pattern in the rapidly growing tumor which consisted mostly of lymphocytes (fig. 9). None of the tissues studied furnished a better example of cellular storage of dye than did this tumor. The lymphocytes contained no dye.

II. Toxicity and lethal dose (LD)

In the toxicity study, the rats which received small doses of chlorazol fast pink showed evidence of some color within a few minutes. This coloring was easily observed in the oral and genital mucosae. With the higher doses the rats were deeply colored within an hour. Large doses of chlorazol fast pink solutions produced a marked diarrhea and one pregnant rat aborted as a result of the treatment. When given intravenously a dose of 0.1 cc. of an 8% aqueous solution per 100 gm. body weight was sufficient to produce death. The results of the intraperitoneal injections are given in table 1. The numbers of rats dead in 24 hours are recorded. There was a fairly uniform increase in mortality with increase in dosage (fig. 2). The LD 50 calculated according to the method of Bliss ('35) was of the order of 0.5 cc. per 100 gm. of rat body weight. As may be seen in figure 2, there was no evidence of a marked decrease in the toxicity of the purified re-crystallized chlorazol

fast pink. In 20-gm. mice the minimum lethal dose was 0.2 cc. of 5% aqueous solution (subcutaneously or intraperitoneally).

At autopsy the mice which had received lethal doses of dye were more heavily stained than those which had been injected with smaller amounts. The stomach, duodenum, and jejunum showed mucosal hemorrhages. In several animals the jejunum contained soft blood clots. In some instances small hemorrhages were also present in the meninges.

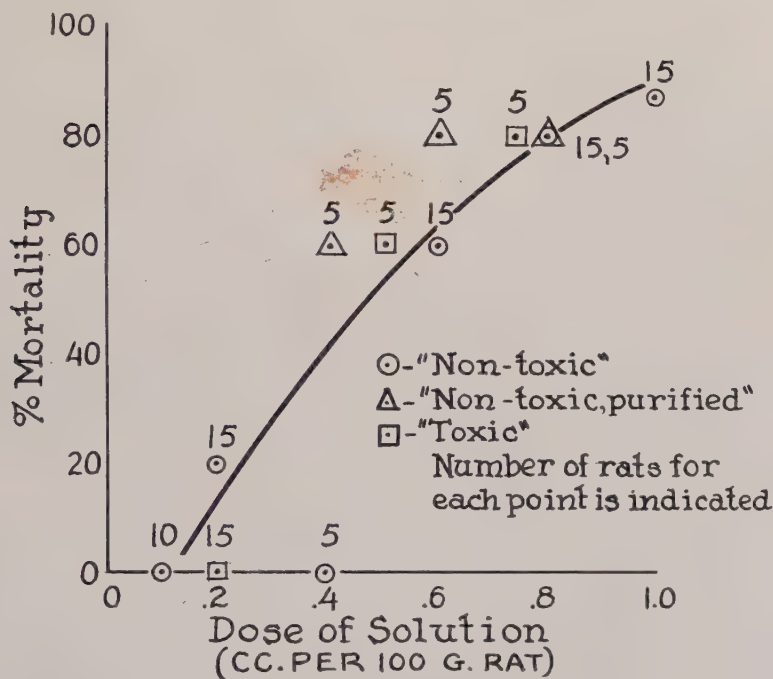


Fig. 2 Toxicity in rats of three fractions of chlorazol fast pink (8% aqueous solutions injected intraperitoneally).

Lethal doses of chlorazol fast pink exerted their most profound effect on the kidney. All of the nephron including the glomerulus and the secretory tubules was severely damaged (figs. 6 and 7). Necrotic cells containing large intracytoplasmic and intranuclear accumulations of dye were observed in all portions of the nephron. The vascular endothelium of the

glomerulus as well as the epithelium of the glomerular capsule contained dye. The collecting tubules were relatively free of dye, although many lining cells had sloughed and some of those remaining in place were swollen and had pyknotic nuclei. A few of the hepatic parenchymal cells were necrotic. In such cells both the cytoplasm and nucleus were heavily stained with chlorazol fast pink. However, definite degenerative changes and necrosis were for the most part limited to the kidney.

III. Effects on coagulation and bleeding times

Table 2 presents the findings relevant to the effects of two different dose levels of chlorazol fast pink on coagulation and bleeding times in mice. The normal in vitro blood coagulation time of the ten mice used in this portion of the experiment averaged 4.6 minutes and the bleeding time 2.6 minutes. Six daily subcutaneous injections of 0.1 cc. of a 1% solution of chlorazol fast pink increased the coagulation time to an average of 15 minutes and the bleeding time to an average of 14.6 minutes. Sixteen hours after a single subcutaneous or intraperitoneal injection of 0.2 cc. of a 5% solution of chlorazol fast pink the average coagulation time had risen to 32.5 minutes and the bleeding time to 37.5 minutes. Not only was coagulation inhibited and the bleeding time greatly prolonged by the dye, but the clots formed within the coagulometer were very fragile and were easily separated and broken by increasing the force of the current of air.

DISCUSSION

I. Vital staining

Bouffard ('06) was apparently the earliest worker to describe in any detail the effects of benzdine dyes when applied to the living animal. Since that time there has accumulated a voluminous literature concerning the reactions of many species to a wide variety of so-called "vital dyes" and to many types of particulate matter (Goldmann, '09; Kiyono,

TABLE 2
Effect of chlorazol fast pink on coagulation and bleeding times in mice

ANIMAL NUMBER	TREATMENT	INJECTION SITE	PERIOD OF DYE ACTION	COAGULATION TIME IN MINUTES		BLEEDING TIME IN MINUTES	
				Previous to dye injections	Subsequent to dye injections	Previous to dye injections	Subsequent to dye injections
S-1a	0.1 cc./diem of 1% CFP* for 6 days	Subcutaneous	6 days	4.5	18.0	2.5	18.0
S-2a	same	same	same	4.5	10.0	2.5	10.0
S-3a	same	same	same	5.0	14.0	2.5	12.5
S-4a	same	same	same	5.0	18.0	3.0	18.0
			Average.....	4.8	15.0	2.6	14.6
C57-1	A single injection of 0.2 cc. 5% CFP	Subcutaneous	16 hrs.	4.0	40.0	2.5	40.0
C57-2	same	same	same	4.0	20.0	2.0	20.0
S-1	same	Intraperitoneal	same	4.5	20.0	3.0	25.0
S-2	same	same	same	4.5	25.0	2.5	25.0
S-3	same	same	same	5.0	45.0	2.5	55.0
S-4	same	same	same	5.0	45.0	2.5	60.0
			Average.....	4.5	32.5	2.5	37.5

* Chlorazol fast pink

'14; Evans and Schulemann, '14; Downey, '17 and '18; Schulemann, '17; Wislocki, '17 and '20; Clark and Clark, '18; McClure, '18; von Möllendorf, '20; Evans and Scott, '21; von Jancsó, '27 and '29; Esaki, '28; Ludford, '28, '30, and '31; Cappell, '29 a, b, c, '30 a and b; Bloom, '38a and b; Jaffé, '38; Mann and Higgins, '38). These references include only a relatively small number of the more comprehensive studies concerned with the storage of dye by animal cells. Cappell (29a, b, c, 30a and b) presented one of the more recent and extensive reports of the intra vitam staining capacity of a large number of dyes in several mammals. Jaffé ('38) gave a very complete review of the subject of vital staining. Aschoff ('24) used the term "reticulo-endotheliale System" which has subsequently been utilized in many instances to designate at least in part the "system of macrophages" which has the ability to store colloidal vital dyes and several types of particulate matter. Relevant to the activity of macrophages is the fundamental work of Metschnikoff ('05) on phagocytosis and immunity.

In this report the term vital staining has been used to denote the intracellular presence of the dye which had been previously introduced into the living animal. Such dye was observed to be present within cells in at least two forms. The first was as a diffuse stain or suffusion which colored the cytoplasm. The terms cytoplasmic "stain" or suffusion were utilized to indicate what was perhaps the dye in an ultra-microscopic particulate or granular state which precedes its segregation or storage as large particles or granules. The appearance of the dye in this condition was amorphous. In so far as could be determined pre-existent cellular structures were not revealed as the result of the dye precipitating on their surfaces or dissolving in their contents. In the second state the dye occurred as distinct cytoplasmic granular accumulations, and this has been termed dye storage or phagocytosis. A third but apparently extracellular collection of dye has been designated as adsorption (fig. 4). This latter phenomenon was most frequently related to the lining cells of the

intranodal lymphatic sinuses and the usual condition was characterized by the presence of dye particles along the peripheral (sinusoidal) borders of these cells. Whether or not adsorption and cytoplasmic suffusions represent antecedent stages in the segregation (Renaut, '07; Evans and Scott, '21; Lewis, '24) and storage of dye could not be determined (see Evans and Scott, '21; von Jancsó, '27 and '29; and Jaffé, '38). Much of the dye which only stained cells was absent subsequent to washing the sections in water for 10 to 30 minutes. Likewise, considerable washing in water removed the dye particles which had accumulated at the periphery of dye-storing cells. In contrast, segregated intracytoplasmic particles and granules of dye were unaffected by the washing procedures. The limited amount of washing in water (2-3 seconds) required for nuclear staining with toluidine blue had little effect on the cytoplasmic staining by chlorazol fast pink, or upon the adsorbed particles. There was also no discernible effect upon the stored or segregated dye. The intracellular storage of "excessively minute" particles has been termed "dypsocytosis" (Evans and Scott, '21). The cytoplasmic dye granules which occur within macrophages vary considerably in size, shape, and arrangement. These variations are said to depend on the dye employed, the dose, and upon the rapidity of the vital staining (von Möllendorf, '20; Evans and Scott, '21; Davies, Wadsworth, and Smith, '30).

Storage of vital dyes by fat cells has received relatively little attention. All of the dyes used in the present study were stored to some degree by fat cells. The large adult "signet ring" fat cells contained dye in the peripheral cytoplasmic ring. A greater amount of dye was present in the same area of smaller fat cells (fig. 3). The lipoblast-like moruloid adipose cells contained cytoplasmic accumulations of dye granules. Both cytoplasmic suffusion (staining) and segregation of dye in granules occurred in large and small fat cells. In general the cells of brown fat particularly that in the perirenal and periaortic areas contained more dye. The more the structure of the fat cell approached the adult "signet ring" type,

the less dye they contained. The fat cells of the interscapular fat "gland" did not contain any dye. However, there were many true macrophages in the perilobular connective tissue of this structure. Washing of the sections in water removed more dye from the fat cells than from the usual macrophages. Wells ('40) and Doan ('40) have discussed the relation of adipose tissue to the reticulo-endothelial system. Dogliotti ('28) and Bremer ('38) have reported some degree of dye storage by fat cells. There are several reports concerning the phagocytic activity of fat cells and the transition of fat cells to macrophages (Goldner, '34a and b; Chang '40). In contrast to the findings of these workers the fat cells which contained chlorazol fast pink (and also trypan blue) were not altered morphologically but were simply several usual types of fat cells which contained dye. During post-secretory mammary involution there is a considerable growth and transition of adipose tissue (Wasserman, '31; Williams, '42a and b). Under these circumstances the roles of fat cells and macrophages are intimately related and the former showed evidence of a type of phagocytic activity (Williams, '42b).

It can be concluded therefore that several types of fat cells store vital dyes. There is also evidence that under certain conditions fat cells are transformed, or undergo a transition to true macrophages (Goldner, '34a and b; Chang, '40). The phagocytic activity of macrophages toward lipids is well known and needs no discussion here.

Several workers have observed that the interstitium of the testis was stained by vital dyes (Goldmann, '09; Evans and Schulemann, '14; Wagner, '23; Esaki, '28; Cappell, '29b). Chlorazol fast pink stained the Leydig cells as well as other cells of the interstitial tissue (fig. 8). However, granular deposits of dye were not clearly evident in the Leydig cells. Furthermore, the dye in the Leydig cells was relatively easily removed by washing in water, whereas the granular deposits of dye present in other cells remained.

Vital staining of the macrophages of the transplanted lymphosarcoma differed in no way from that occurring in the

phagocytic cells present in the reticulum of lymph nodes. The tumor used in these experiments was of mediastinal origin, probably thymic. It represents the type of mediastinal lymphosarcoma which develops in several strains of mice as the result of treatment with estrogens (Gardner, Kirschbaum, and Strong, '40). The tumor had been through sixteen transplant generations at the beginning of this experiment and since that time has gone through eight more generations. There was an abundance of dye containing macrophages in the tumor. These cells had no particular anatomical distribution. The lymphoid elements of the tumor were not stained by the dye.

In the kidneys of animals which had received non-toxic doses of chlorazol fast pink the cytoplasm of the lining cells of the proximal convoluted tubules contained large amounts of dye in granular form (fig. 5). These renal cells were the only normal parenchymal elements which stored any of the dyes. The earliest workers with vital dyes noted this condition. Goldmann ('09), Ludford ('28), Cappell ('29b), and others presented complete descriptions of dye storage in the kidneys of mammals. Wislocki ('17) reported that the endothelium of the renal portal system of the mesonephric kidney of the teleosts stored trypan blue in considerable quantity, but that the tubular epithelium contained very little dye. The kidney of the foetal guinea pig excretes trypan blue and the dye is stored in the lining cells of the proximal, convoluted tubules (Wislocki, '20). The reactions of the kidney to the lethal doses of chlorazol fast pink will be discussed later.

The dye was present in the bile as well as in the urine. However, gross examination indicated that little if any dye was present in the feces. In fact, the colon and rectum contained only slight traces of dye. The lymphatic vessels and nodes of the intestine were filled with dye. There are several reports which describe in detail the mode of excretion of vital dyes and the quantitative aspects of excretion and storage (Smith, '30a, b, c; Victor, Van Buren, and Smith, '30; Davies, Wadsworth, and Smith, '30).

II. Toxicity and lethal dose

From the toxicity data (table 1) the amount of an 8% aqueous solution of chlorazol fast pink required to kill the average 100-gm. rat is calculated as of the order of 0.5 cc. No difference was observed in the toxicities of the unpurified "toxic" fraction and the purified "non-toxic" fraction. This finding is unexplained. It may be that certain highly toxic substances were contaminants. It is also possible that investigators who have found highly toxic chlorazol fast pink preparations may have injected imperfect solutions so that particulate matter, undissolved crystals of chlorazol fast pink, were injected. The 8% solution of chlorazol fast pink has a very deep color so that it is difficult to ascertain the completeness of solution. The diarrhea which was observed following intraperitoneal injection is probably one of the symptoms of chlorazol fast pink poisoning. Specific evidence of this toxicity effect is found in the hemorrhages observed in the intestinal mucosa.

The question of dosage for experiments in which animals are to be given chlorazol fast pink to prevent the formation of blood clots in arterial or venous cannulae may be in part answered from the present data. Thus, 1 cc. of an 8% aqueous solution per 100 gm. body weight intraperitoneally killed all the rats. However, 0.1 cc. per 100 gm. body weight killed none. This is certainly a safe dose. From our experience in using chlorazol fast pink as an anticoagulant in cats and rabbits the importance of giving small injections when the solution is given intravenously should be emphasized.

The morphological reactions to lethal doses were most evident in the kidney. Damage to the whole nephron including the glomerulus and its vascular elements was indicated by necrosis of epithelial and endothelial elements and by the presence within these cells of an amorphous mass of dye which filled the cytoplasm and stained the nucleus (figs. 6 and 7). Washing in water did not remove this dye from the sectioned material, and the cytoplasm and nuclei would not "take" any of the usual stains. At non-toxic dose levels storage was limited to granular accumulations of dye in the cytoplasm of

the lining epithelium of the proximal tubules and of the glomerular capsule. That wide-spread cytoplasmic and nuclear staining by vital dyes constitutes evidence of necrosis seems to be a valid conclusion with which the reactions of the epithelium of the nephron to lethal doses of chlorazol fast pink conform (Lewis and Lewis, '15; Downey, '17; Lewis, '21; Lewis and McCoy, '22; Prigosen, '21 and '24; Sabin, Doan, and Cunningham, '25; Cappell, '29a; Belkin and Shear, '37; Shear and Belkin, '37). A very limited number of hepatic parenchymal cells showed this cytoplasmic and nuclear staining. In addition to these histopathological changes, there was gross evidence of musosal hemorrhages in the stomach, duodenum, ileum, and meninges. Soft blood clots were present in the lumen of the ileum.

III. Coagulation and bleeding times

Intraperitoneal and subcutaneous application of aqueous solutions of chlorazol fast pink definitely increased bleeding and coagulation times (table 2). The control bleeding and coagulation times were of slightly greater duration than those previously reported for mice (Copley and Lalich, '42; Lalich, Lalich, and Copley, '43). However, different methods were used for the present determinations and the effects of the dye were so pronounced that the results are significant (table 2). It has been stated that intravenously injected azo-dyes prolonged blood clotting by inhibiting the effect of calcium and thrombokinase on the fibrinogen-prothrombase complex (Huggett and Silman, '32; Huggett and Rowe, '33a and b). The latter workers reported that chlorazol fast pink applied intravenously to rabbits was twice as effective as the heparin used, and also that the minimum lethal doses of chlorazol fast pink and of heparin were the same. As the result of their study of several substances they concluded that the dyes which are most effective as anticoagulants are ". . . disazo direct dyes prepared from tetrazotised diamines coupled with aminonaphthol and sulphonic acid." Huggett ('34) stated further that chlorazol fast pink and certain other azo-dyes prolonged

coagulation time "... by inhibiting the action of the enzymes thrombokinas and thrombase" in converting the prothrombase to the plasma to thrombase and the plasma fibrinogen to fibrin. However, Barratt ('37) reported that the "action of the anti-coagulants NaCl, chlorazol and heparin in delaying the coagulation of a mixture of fibrinogen and thrombin is exerted upon thrombin; no evidence of any action upon fibrinogen was observed". Quick ('37) using a dye identical with or closely related to chlorazol fast pink was unable to determine definitely which component of the clotting mechanism was acted upon by the anticoagulant azo-dyes. Jorpes ('35) stated that what seems to be the active anticoagulant principle of heparin "... bears a resemblance to all the synthetic anti-coagulants which are sulphonic or polysulphonic acids." Later it was reported (Sterner and Medes, '36) that several sulphur containing compounds including cysteine inhibited clotting when added to whole blood; and further, that cysteine and methionine administered orally or intravenously to the human prolonged both bleeding and coagulation times. Jorpes and Bergström ('39) have also investigated the relation of the sulphur content of heparin and other materials to anticoagulant activity. In mice, excessively large doses of heparin prolonged both the bleeding and coagulation times, whereas smaller amounts effected only the coagulation time (Copley and Lalich, '42). Also, no correlation existed between coagulation times, bleeding times, and prothrombin times subsequent to the oral administration of 3, 3'-methylene-bis (4-hydroxycoumarin) at several dose levels (Lalich, Lalich and Copley, '43). These workers observed that some of the mice showed normal coagulation and prothrombin times, but a prolonged bleeding time; while in other mice prolonged coagulation and prothrombin times occurred concomitantly with a normal bleeding time.

Therefore, certain azo-dyes, many of which have been used for vital staining, are effective as anticoagulants when injected intravenously, subcutaneously, intraperitoneally, or applied locally. However, the mechanism by which these dyes inhibit

the coagulation of blood has not yet been clearly demonstrated. When injected in amounts which are well tolerated by mice and rats the prolongation of bleeding and coagulation times seemed to be the only deleterious effect of the dyes.

SUMMARY AND CONCLUSIONS

1. When injected subcutaneously or intraperitoneally in rats and mice chlorazol fast pink was stored by the cells of the system of macrophages in a fashion comparable to the other dis-azo coloring matters. Morphologically normal cells showed (1) cytoplasmic storage or segregation of the dye in granular form; (2) an adhesion or adsorption of the dye granules at the external surface of macrophages, particularly those lining the sinuses of lymph nodes; and (3) cytoplasmic suffusion of the dye. The lining cells of the renal proximal convoluted tubules contained dye in granular form.

2. In adipose tissue, particularly that located in the perirenal and periaortic areas, dye was stored in the peripheral cytoplasmic ring of larger fat cells and in the cytoplasm proper of smaller fat cells.

3. The toxicity of 8% aqueous solutions of chlorazol fast pink, purified and unpurified, was tested by intraperitoneal injections in 115 adult albino rats. The LD 50 was of the order of 0.5 cc. per 100 gm. body weight. No difference in toxicity was observed between purified and unpurified samples. The minimum lethal dose for 20-gm. mice was 0.2 cc. of a 5% aqueous solution (subcutaneous or intraperitoneal). Lethal doses of chloral fast pink caused petechial hemorrhages in the mucosa of the gastrointestinal tract and to a lesser extent in the meninges. The proximal convoluted tubules of the kidneys showed the greatest degree of damage. The nuclei of the lining cells were stained and the cytoplasm was filled with an amorphous mass of dye. The epithelium of the glomerular capsule and the endothelium of the glomerular capillaries showed a similar type of necrosis and damage. Many of the lining cells of the collecting tubules were necrotic, but were relatively unstained by the dye.

4. All of the doses used caused a rapid coloring of the animal. Dye was excreted by the kidneys and in the bile. The gastrointestinal tract contained some dye in the duodenum and jejunum. The ileum and colon were relatively free of dye and little was present in the feces. The lymph nodes of the gastrointestinal tract contained more dye than did any other tissue or organ. Trypan blue was distributed and excreted in a similar fashion.

5. In mice subcutaneous and intraperitoneal injections of chlorazol fast pink inhibited the *in vitro* coagulation of blood and extended the bleeding time. Six daily subcutaneous injections of 0.1 cc. of a 1% aqueous solution of chlorazol fast pink increased (by the seventh day) the time required for *in vitro* coagulation an average of 10.3 minutes beyond normal, and the bleeding time 12 minutes beyond normal. Sixteen hours after either a single subcutaneous or intraperitoneal injection of 0.2 cc. of a 5% aqueous solution of chlorazol fast pink the coagulation time increased from an average normal of 4.5 minutes to 32.5 minutes, and the bleeding time from an average normal of 2.5 minutes to 37.5 minutes.

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PLATE

PLATE 1

EXPLANATION OF FIGURES

3 Perirenal fat of mouse vitally stained with chlorazol fast pink. Note granular accumulations and cytoplasmic suffusions of dye. $\times 450$.

4 Lymph node of mouse. Large amounts of chlorazol fast pink present within and absorbed to the luminal borders of the cells lining the intranodal sinuses. Nuclei stained with toluidine blue. $\times 775$.

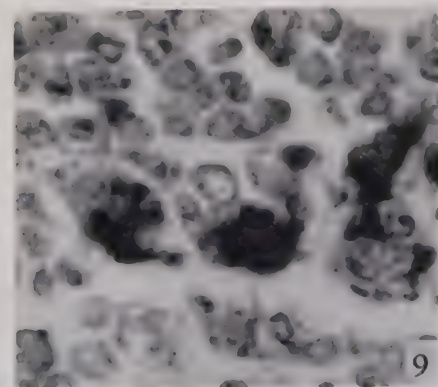
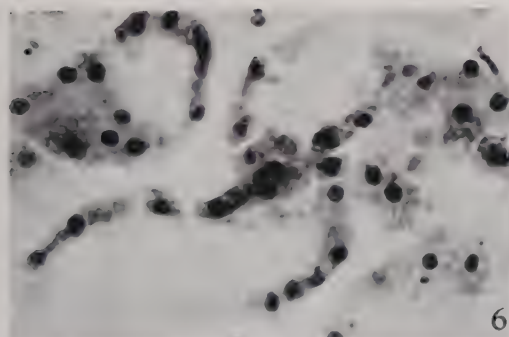
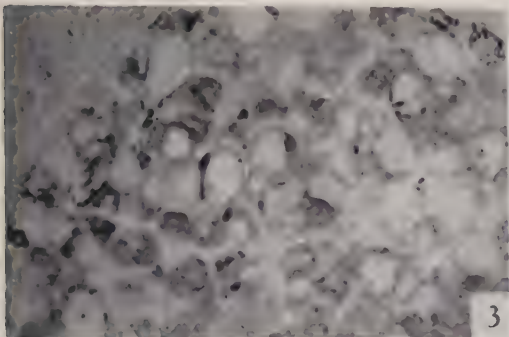
5 Kidney of mouse which received a non-toxic dose of chlorazol fast pink. Dye in granular form is present in the cytoplasm of the epithelium of the glomerular capsule and of the proximal convoluted tubules. $\times 400$.

6 Kidney in rat which received a lethal dose of chlorazol fast pink. There is a generalized staining of cytoplasm and nuclei in the proximal convoluted tubules. Note the amorphous appearance of the dye. $\times 400$.

7 Similar to figure 6 except that the section was stained with Ehrlich's hematoxylin. Note the presence of the vital dye in the cytoplasm and nuclei of necrotic epithelial cells. $\times 400$.

8 Interstitial tissue of mouse's testis. Note inclusions of chlorazol fast pink in many cells, while others are lightly stained. Nuclei stained with toluidine blue. $\times 600$.

9 Dye containing macrophages in a transplanted lymphosarcoma (mouse). Nuclei stained with toluidine blue. $\times 925$.



THE APPEARANCE DURING ONTOGENY OF A SUTURE BETWEEN PREVIOUSLY FUSED DERMAL BONES

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The premaxillae of the salamanders of the family Plethodontidae consist of anterior dentigerous rami and posteriorly directed nasal spines that either separate the nasals from one another or underlie them (Dunn, '26, p. 40). Drawings of premaxillae from several plethodontid salamanders are to be found in Wilder ('24, pp. 539-541).

During a preliminary comparative survey of the skulls of *Pseudotriton* and *Gyrinophilus*, two closely-allied boletoid-tongued genera of the Plethodontidae, it was noticed that in *Gyrinophilus* the dentigerous ramus of the premaxilla was sometimes fused with its fellow across the midline and sometimes a suture was present between them. In all cases observed the nasal spines of the premaxillae were well separated. It was thought desirable to offer the present incomplete account because of the difficulty that now exists in obtaining material adequate for the histological study indicated below.

The material upon which this note is based includes 12 dermestid-cleaned *Gyrinophilus* skeletons in the collection of Mr. Joe A. Tihen (nos. 719, 1043, and 1046-55) and eighteen specimens of *Gyrinophilus* cleared and stained with alizarin red, following the procedure of Williams ('41, p. 23), by Miss Marion Ludwig of the University of Rochester.

The observation of greatest interest in this material is that the rami are fused in the smaller specimens but are separated from each other by a suture in the older individuals. We are here dealing with a case in which a suture appears, during

ontogeny, in the region where paired dermal bones have been fused. This is the reverse of the usual direction of ontogenetic changes among the dermal bony elements of osseous vertebrates in which fusion of bones and obliteration of sutures accompanies increase in age. Table 1 summarizes the findings on the available material.

TABLE 1

Relationship between the size (age) and the presence of a suture between the dentigerous rami of the premaxillae in twenty-seven specimens of Gyrinophilus.¹

HEAD-TRUNK LENGTH IN MILLIMETERS ²	DENTIGEROUS RAMI OF THE PREMAXILLAE	
	Number fused	Number separate
31- 35	1	0
36- 40	1	0
41- 45	1	0
46- 50	2	0
51- 55	0	0
56- 60	1	1
61- 65	0	1
66- 70	0	3
71- 75	1	1
76- 80	0	1
81- 85	0	2
86- 90	0	1
91- 95	0	5
96-100	0	3
101-105	0	1
106-110	0	1

¹ Although thirty was given as the number of specimens examined only twenty-seven are accounted for in this table. This is due to the fact that the material studied included one dissociated skeleton and two cleared and stained skulls from which no accurate estimate as to head-trunk length could be obtained.

² The head-trunk length measurement given here is a record of the distance from the anterior end of the skull to the first vertebra with a haemal arch.

The report of such a novel phenomenon in a field that has received so much detailed attention by numerous competent investigators might incline one to suspect that some error of fact or interpretation may have been incorporated in the report. Places where such errors might appear are discussed immediately below.

Are the observations on suture absence and presence reliable? In some of the larger cleared salamanders the suture between the two rami is so wide that a narrow slit is present through which objects can be clearly seen. In favorable younger larvae the fused rami seem to be composed of material that is homogeneous throughout. When the smaller cleared alizarin red specimens are viewed with transmitted light the premaxillary rami are found to be translucent and the structure in the midline does not seem to differ from that in the remainder of the fused rami. A median tooth is often present in small examples. In an older specimen with separate rami a median tooth seemed to be sloughed off and lying free in the soft tissue near the midline.

Are the larval premaxillae actually bone? In the smallest dry skeletons the premaxillae are composed of bone and certainly not of desiccated cartilage. The premaxillae take the alizarin red stain and do not take the toluidine blue.

Are the larvae and the adults the same species? The major part of the stained and cleared material was collected at one time (September 27, 1942) in one short stretch of stream in Allegany State Park, New York, by Dr. S. C. Bishop, Misses Hulda Gross and Barbara Leonard, and the present writer. The larvae and adults were independently identified in the field by Dr. Bishop and the writer as *Gyrinophilus p. porphyriticus* and this identification was later confirmed by laboratory study. *Pseudotriton r. ruber* is the only other salamander species that has ever been taken in the Park area and with which *Gyrinophilus* could conceivably have been confused. Only three specimens of *Pseudotriton* are known from the Park (Bishop, '41, p. 275), as contrasted with many examples of *Gyrinophilus* (idem., p. 257), and these were collected in a section of the Park remote from that in which the present study material was taken. Finally, Dr. Bishop has been quite anxious to secure *Pseudotriton* material from the Park and carefully goes over larval *Gyrinophilus* specimens with that in mind. Thus it seems quite unlikely that any *Pseudotriton* specimens are included in the series studied.

It may also be suggested that two species of *Gyrinophilus* coinhabit the stream; one with fused premaxillae and one with separate premaxillae. This would lead to the untenable conclusion that species A is known only as adults and species B only as non-neotenic larvae.

Are the larvae with fused rami examples of an anomalous year group? The size range of the larvae examined would indicate that they compose 3-year groups (*idem.*, p. 250). Certain other collections (October 18, 1941, March 9, 1942, and June 7, 1942) agree with the September, 1942, material.

This observation indicates the need of an histological study of the larvae and transforming individuals of *Gyrinophilus*. Serial cross-sections of the snout region of such material should be prepared and observations made along the midline. A search should be made for cells that might function as osteoclasts.

In summary, this note reports the observation of fused premaxillary rami in smaller (younger) individuals of the salamander, *Gyrinophilus p. porphyriticus*, and separate premaxillary rami in larger (older) specimens. It is thought that the report of a suture appearing, during ontogeny, in the midline, across which paired dermal bones had been fused, is novel to present morphological literature.

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A CASE OF DUPLICATION OF THE HYPOPHYSIS IN A 10-MM. PIG EMBRYO

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ONE PLATE (THREE FIGURES)

Many anomalies of the hypophysis have been observed and recorded—notably in the 40-mm. pig (Holt, '21); in human fetuses (Covell, '27); in the two-toed sloth and the three-toed sloth (Wislocki, '38); in three species of whales (Valso, '34; Geilung, '35; Wislocki and Geilung, '36); in the porpoise (Wislocki, '38); and in the manatee (Oldham, McCleery, and Geilung, '38).

In each of these presentations, however, the anomaly described is congenital absence of a portion of the hypophysis, while the condition reported here concerns not the absence of a part, but the duplication of a part—in fact a potentially double hypophysis. So far as I can ascertain, such a condition has never been recorded before.

The extraordinary features observed in this 10-mm. pig embryo consist of a duplication of two parts—namely the diencephalic floor and the hypophysis. It is also observed that the anterior neuropore, which is normally closed at this stage of development, is still patent.

In the interpretation and explanation of these findings, it is best to start with the anterior neuropore. The reason for the patency of this structure is probably the increased bulk in the median region, which in turn would cause a delay in conrescence.

I believe that the increased bulk in the media region is not limited to this area alone, but is also found in the floor of the diencephalon.

The neural tube in expanding in the long axis produces a 'foreward crowding' (Kingsbury and Adelman, '24). This would cause a pivoting on the predestined fulcrum, the primitive infundibulum. Due to the increased bulk of the diencephalic floor, this fulcrum was unable to withstand the increased pressure and became ironed out laterally. With the release of pressure, the primitive infundibulum became divided. The rotation of this small segment of the brain floor produces a small depression or outpocketing in the brain floor which becomes the pars neuralis (Gilbert, '35). In this case, therefore, there would follow a duplication of the pars neuralis.

There is a persisting adherence of the surface ectoderm to the neural tube over a limited median area on the ventral surface of the head (Gilbert, '35). As this median area had been previously divided with the resulting duplication of the pars neuralis, it follows that there would also be a doubling of the hypophysis. Thus a doubling of Rathke's pouches.

It is a pleasure to acknowledge the help and guidance of Dr. E. M. Shearer of the department of anatomy of the New York University College of Medicine.

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PLATE 1

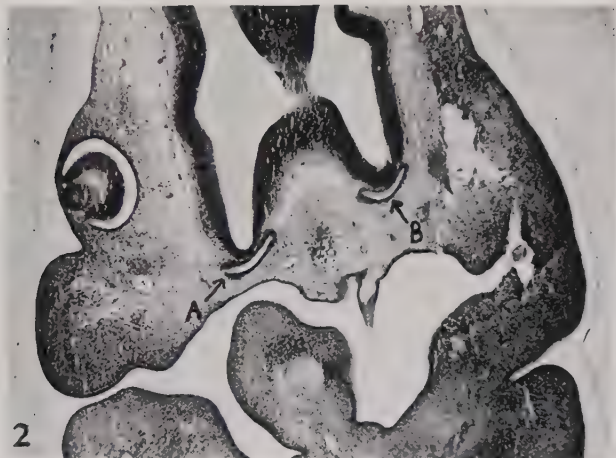
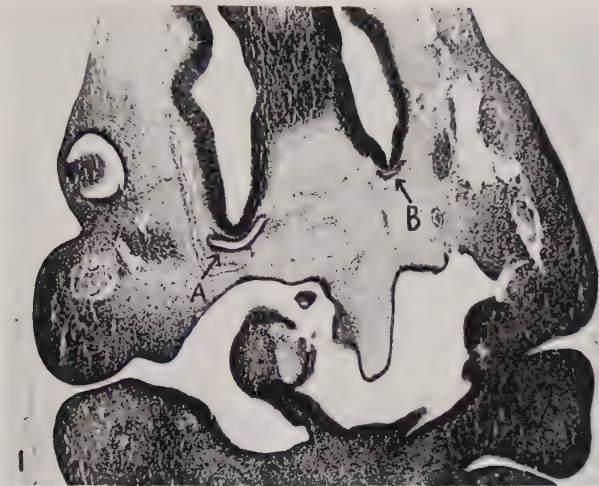
EXPLANATION OF FIGURES

Selected sections ($13\ \mu$) from a serially sectioned 10-mm. pig embryo, illustrating doubling of the hypophysis. ($\times 31$)

1 Section of the diencephalon showing one hypophysis at (A) and first indication of second hypophysis at (B).

2 Section 26 μ . below the preceding section. This shows the diencephalon and complete hypophyses at (A) and (B).

3 Section 65 μ . below the preceding section. This shows at (A) evagination of the pharynx to form one Rathke's pouch. Evagination at (B) although not shown in these sections, proceeds as in (A).



VASCULAR CHANGES IN THE RABBIT UTERUS AND IN INTRAOCULAR ENDOMETRIAL TRANSPLANTS DURING PREGNANCY¹

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Although there have been numerous studies of vascular changes in the primate uterus, particularly through the menstrual cycle (Bartelmez, '31, '33; Hartman, '29a, b, '32; Markee, '33, '36, '38; Daron, '36, '37 and others) relatively few studies have been made of these changes in the rodents. In the pregnant rat Goldman ('09) worked out the general pattern of circulation in the uterus in his attempts to determine the anatomic pathways involved in the production of uterine extravasates. Long and Evans ('20), although not concerned with vascular changes, described accurately the appearance of the placental sign. More recently Stafford ('30) injected india ink into blood vessels of rodents and studied serial sections of the injected uteri. Working with a few guinea pigs and rabbits, but primarily with the rat, he described cyclic changes in the vascular pattern of the rat uterus and stated that similar, though less intense changes occurred in the gravid uterus of rabbits. Westman ('31) using a similar technique, studied only the effects of extirpation of the corpus luteum on vascular changes in the rabbit uterus. In 1929 Markee described rhythmic variations in the vascularity of the uterus of the guinea pig during the oestrous

¹ This study was begun in the Department of Anatomy, Stanford University and was completed in the Department of Zoology, University of California, Los Angeles. The author expresses his thanks to Prof. J. E. Markee, Department of Anatomy, Stanford University, for suggesting the problem and for suggestions and criticisms during its execution.

cycle and in 1931 and 1932 he described rhythmic vascular changes in the nonpregnant rabbit uterus. In 1934 Markee and French described vascular changes in intraocular endometrial and myometrial transplants in pregnant rabbits.

The studies reported herein concern uterine vascular changes during pregnancy as seen in fixed tissues and it must be admitted that what is observed in fixed material may differ from what is present in living tissues. Shrinkage and contraction during fixation certainly alter the tissue. Moreover, during death of the animal relaxation of the muscular wall of certain large blood vessels must occur, resulting in a fall in blood pressure and a pooling of blood in the abdominal organs, probably in the liver. Thus, blood normally present in the uterus may leave the organ. These criticisms apply not only with reference to the uterus but to endometrial transplants and all other organs as well. But, as will be pointed out, vascular changes observed in sectioned uteri and endometrial transplants in the course of this study are in strikingly good agreement with the findings of investigators who employed a physiologic approach or who observed living tissues.

MATERIALS AND METHODS

The animals used in this study were described in a previous communication (Krichesky, '42) and consisted of fifty-nine adult female rabbits which received intraocular endometrial transplants after the method of Markee ('40). In forty-eight, or 81%, the transplants were successful. Not less than 10 days were allowed for vascularization and organization of the transplants before these animals were mated. At selected intervals after mating, varying from 1 day postcoitum to 12 days post partum, the animals were sacrificed by intravenous injection of 20 to 40 cc. of air into the marginal ear vein. In addition, four unoperated animals were used, two sacrificed on the day of delivery and one each on the first and second day post partum. Thus a total of fifty-two animals provided material used in this study.

Uterine transplants and small segments of the occupied and unoccupied² uterine horns were quickly removed and placed in Bouin's and Zenker-formol fixing fluids. In animals sacrificed in the later stages of pregnancy tissues were taken only from between implantation sites. In eight animals killed in late stages of pregnancy, tissues were also fixed in Regaud's fluid and in acetic-osmic-bichromate mixture. In two animals, representing the first and second day after delivery uterine material was removed and fixed in Müller's fluid as well as in chilled alcohol after freezing in situ.

The majority of the material collected was dehydrated and cleared in dioxan, the remainder by the usual alcohol method. Most of the tissues were imbedded in paraffin with a melting point of 56–58°C.; a few were imbedded in celloidin. The stains employed were Delafield's hematoxylin and eosin, Delafield's hematoxylin and eosin-azure, iron hematoxylin and triosin, and Heidenhain's Mallory-azan.

OBSERVATIONS

Vascular pattern of the nonpregnant rabbit uterus

No complete studies of the vascular supply of the rabbit uterus was undertaken but from observations in the course of this study, the following general statements can be made: arteries enter the uterine horns from the mesometrium and take a twisted course until they lie between the longitudinal and circular layers of muscle, encircling the latter and giving off numerous small branches to both. Some of these small arteries penetrate the circular muscle and pass into the basal zone of the mucosa in which many of them continue longitudinally. From these and from the circular arteries, arterioles and long capillaries arise, some of which pass radially toward the epithelium. The rabbit endometrium lacks the characteristic coiled arteries that supply the endometrium of the primate (seen in the human and rhesus monkey, Daron, '36).

² Surgical removal of a portion of one horn for implantation results in sterility of that horn. In the occupied horns tissues were taken only from between placentae.

Venous drainage from the uterus follows a similar but reverse path from that of the arterial supply. This also differs from the venous pattern of the monkey since in this form there are large venous sinuses with afferent and efferent channels (Daron, '37) which do not resemble a branched tree as in the rabbit.

Since this study deals only with vascular changes, only size, distribution and contents of endometrial vessels are discussed here. Numerous vessels of moderately large size lie in the basal region of the mucosa while the superficial regions appear much less vascular, containing only a few small arterioles and capillaries which make up the most numerous elements. The majority of the capillaries in the longitudinal folds of the endometrium appear to be contracted, since their endothelial cells are short and relatively thick. In fixed material, but probably not in the living, most of these superficial capillaries contain no blood cells.

Observations on the uterus during pregnancy

First through eighth day. From the day of mating through the fourth day postcoitum, the area of the endometrial vascular bed is markedly increased. Large vessels become more numerous in the deeper regions of the mucosa and the vascular bed of the superficial regions becomes more extensive. In contrast to the condition in the nonpregnant uterus, the most striking change is the presence of numerous blood cells in the vessels of the superficial mucosa, approximately 75% of these channels being almost completely filled with blood cells. In addition, a plexus of capillaries has opened up or developed in this region, parts of it lying in intimate contact with the epithelium.

From the sixth through the eighth days of pregnancy two additional changes occur. First, the growth and invagination of uterine glands is very extensive, crowding out the connective tissue between them so that frequently the region between two adjacent glands is occupied only by a single

capillary and its supporting connective tissue. Second, by the sixth day there occur apparently new, moderately large vessels, primarily in the basal region of the endometrium. About one half of these are veins and venules, about one quarter are lymphatics, and the remainder are dilated capillaries. The majority of these vessels, whether veins, capillaries or lymphatics, are lacking in formed blood elements, and are filled with a stainable coagulum only. These large channels are most numerous by the sixth day and by the eighth day some of them appear to be crowded out by the growing glands.

Tenth through twenty-second day. Between the tenth and twenty-second day of pregnancy there is a gradual increase in area of the vascular bed. More large vessels appear, so many of them in some regions that little space is occupied by connective tissue. More dilated venules and capillaries appear throughout the stroma, approximately one third of them containing only a clear, stainable coagulum. Only the vessels nearest the epithelium include blood cells. Some lymphatic channels contain lymphocytes and granular leukocytes and only occasionally are large macrophages present.

The presence of large blood channels containing scarcely any blood cells probably indicates that, in spite of the increased area of the vascular bed, there is a reduced blood flow. An extensive vascular bed is not always associated with a larger volume of blood flow since the volume of flow is determined by the rate of flow as well as by the cross sectional area of the channel. Constriction of the veins might fill the capillaries and venules by damming back the blood, thereby decreasing the volume of blood flow, but venous constriction does not explain the absence of blood cells in the lumina of these vessels. Constriction of the arterioles would reduce the blood flow but does not explain the condition observed in the uterine vessels in which only few blood cells are present, and crowded to one side of the lumen. It has been suggested (Markee, unpublished) that this condition may be the result of "skimming," a phenomenon in which arterioles are constricted sufficiently to prevent passage of blood cells but not

plasma, so that in effect the plasma is filtered through a plug of blood cells proximal to the site of constriction. This would result in a slow movement of plasma through the capillary bed and would permit corpuscles passing the point of constriction to settle out. Such a view finds support in the findings of Stafford ('30) who observed that india ink injected into the blood stream of the rat was blocked in the arteries of the myometrium even though the area of the endometrial vascular bed was increasing.

Twenty-third through twenty-seventh day. During this 5-day period the most striking change in the vascular bed is a diminution of its area. Large vessels, some filled, some partly filled, and some entirely devoid of blood corpuscles become progressively smaller and less numerous. As in earlier stages, the largest vessels are always limited to the basal region of the endometrium.

Twenty-eighth to day of delivery. From the twenty-eighth day until parturition three distinct modifications of the vascular bed occur in the endometrium. One of these is a gradual enlargement of the venules and capillaries until they occupy nearly all the space between the epithelium and the muscularis. By the thirtieth day almost all of them are completely filled with blood cells, indicating a marked increase in the concentration of erythrocytes in the circulating stream through the endometrium. This striking change almost certainly explains the fact that the color of intraocular endometrial transplants (Markee and French, '34), and the color of the uterus in situ in normal unaesthetized live rabbits as seen through a glass window in the abdominal wall (Markee, unpublished), is most intense in the 3 days preceding delivery. This process apparently reaches its peak at the time of delivery, for in animals sacrificed 4 to 6 hours after delivery, visible blood channels are smaller in size, less numerous and many of their lumina contain no blood corpuscles.

The second change occurring after the twenty-eighth day and continuing until 2 days post partum, is the tremendous increase in the size of lymphatic channels. Some are rounded

in cross section while others are irregular; some contain leukocytes and macrophages while others are devoid of cells and stainable fluid. The latter are not empty since it is impossible to conceive of spaces existing in any living tissue; furthermore, their distended appearance is indicative of a fluid which is either nonstainable or is dissolved out during fixation.

A third conspicuous change in the endometrium after the twenty-eighth day of pregnancy occurs throughout the endometrial stroma and takes the form of cavities of various sizes lacking an endothelial lining. The majority of these lie in the basal portion of the mucosa. Their outlines are irregular and their walls appear to be composed of cells and naked intercellular or ground substance. These lining cells resemble stroma cells more closely than endothelial cells, consequently it is assumed that in the cavitation process the intercellular substance is forced away from one cell surface leaving that surface exposed to the cavity.

In the 2 days preceding delivery the majority of these "tissue spaces" contain stainable coagulum, leukocytes, macrophages, and occasionally giant cells characteristic of this stage of pregnancy. Free ends of fine collagenous fibrils project into them and in a few instances small blood vessels pass through them. Similar "spaces", which, however, lack a stainable coagulum, also appear during this period and increase in number for the next 4 days.

No single satisfactory interpretation for these so-called tissue spaces can be given. The fact that the larger more irregular ones having the appearance of torn places are most numerous adjacent to the muscularis suggest that these are artifacts due to muscle contractions probably during or preceding fixation. But those farther from the muscularis are rounded and regular in outline and it is possible that they normally are present in living tissue. Their presence in the living tissue is suggested by the fact that when these spaces are abundant, lymphatic channels and capillaries also appear to be empty, indicating that all three contain a similar sub-

stance which is either nonstainable or is dissolved out in the preparation of the sectioned material. No matter what the nature of these so-called tissue spaces, they appear in greatest abundance only 2 days preceding and 2 days following parturition.

Day of delivery to twelve days post partum. On the day of delivery and immediately following, there is a marked reduction in the size and number of dilated blood vessels and by the third day post partum, there occurs a marked reduction in size and number of the tissue "spaces". The lymphatics, however, remain large until 8 days post partum.

After the eighth day post partum, small vessels mostly filled with blood cells, are found throughout the superficial portion of the mucosa while the deeper layers contain arteries, veins and lymphatics of moderate size. Thus by the eighth day, the vascular bed is nearly returned to the nonpregnant condition and is fully restored by the twelfth day post partum.

Vascular changes in endometrial transplants

The changes in the vascular pattern in intraocular endometrial transplants are strikingly similar to those in the uterus in situ. All of the following changes in the transplants agree in all essential details with those already described for the uterus: (1) Hyperemia occurs through the third and fourth days after mating. (2) Thereafter there is a gradual increase in size and number of visible blood vessels until the twenty-second day. The majority of these contain a few red blood cells. (3) The area of the vascular bed decreases from the twenty-third to the twenty-eighth day. (4) After the twenty-eighth day the area of the vascular bed increases again until the day of delivery. As in the uterus in situ, this last increase is accompanied by a great concentration of erythrocytes in the channels. (5) After delivery there is a rapid regression until the area of the vascular bed approaches the normal (nonpregnant) condition between the eighth and twelfth day post partum. Thus all of these changes in the vascular pattern of the transplanted endometrium during

pregnancy occur simultaneously and in the same direction with those of the uterus in situ.

There are, however, some few differences in the vascular pattern of the endometrium in the two sites. Characteristic distribution of vessels between the superficial and basal regions of the mucosa in the transplants cannot be determined since in most cases the normal relationships of the tissue of the transplant are altered by transplantation. The coagulum filled vessels characteristic of the latter stages of pregnancy are not as large or as numerous in a transplanted uterus as in a uterus in its normal location.

But in spite of these few differences, the endometrial vascular pattern in the two locations is remarkably similar. The anastomosis of the cut vessels of the transplants with iridial vessels would lead one to suspect alterations but the manipulation of the tissue, transplantation to an abnormal location, and anastomosis with iridial vessels fail to alter the reactions of transplanted endometrium. Moreover, attempts to find alterations in vascular changes in the two locations have been unsuccessful. Markee ('32) has been unable to find them even with the use of abdominal windows, local anesthesia, and direct observation of endometrium in situ through a uteroscope in unanesthetized guinea pigs and rabbits (Markee, unpublished). Not only has no one reported alterations of vascular reactions in transplanted endometrium but all of the changes reported by Markee were first seen in intraocular endometrial transplants and subsequently were confirmed in the uterus in situ.

DISCUSSION

The sequence of vascular changes observed in sectioned uteri and transplants are in good agreement with the findings of investigators who employed a physiologic approach or who observed living tissues. It has been reported by Markee ('31, '32) and confirmed by Fagin and Reynolds ('36), Newman ('32, '34), Markee and French ('34), and MacLeod and Reynolds ('38), that a maximal hyperemia of the endometrium

occurs within 30 minutes after injection of oestrogens into normal or ovariectomized guinea pigs and rabbits. Markee ('31, '32) and Markee and French ('34) made these observations in unanesthetized rabbits, in normal rabbits through an abdominal window, and in intraocular endometrial transplants. Moreover, they have graphically demonstrated that the hyperemia following the mating of normal female rabbits results in an increase in color of the transplants for three or four days. The histologic findings reported here (an increase in the number of dilated vessels nearly filled with erythrocytes) are in complete agreement with their observations.

Markee and French ('34) report that the color of intraocular endometrial transplants is reduced after the fourth day and reaches a low point at approximately the seventh day. The reduction in size and number of the blood vessels in the superficial portion of the mucosa during this time, as reported here, may explain the decreased color observed by them.

Markee and French find a slight but progressive increase in color of endometrial transplants from the seventh to approximately the fifteenth day, followed by an increase in color intensity from the eighteenth until the twenty-fifth day. The changes observed in sectioned material during this period are, (1) increase in size and number of dilated vessels, and (2) a marked reduction in the number of blood cells in them. The gradual increase in color of the transplants may be due to a more effective increase in size and number of vessels than to a reduction in the number of cells in the vessels. It is impossible to evaluate the effect of factors 1 and 2 above because of the variation which exists in different animals and in different sections from the same animals.

The almost complete absence of erythrocytes from the lumina of the blood vessels during this stage of pregnancy probably is due to the action of the corpus luteum hormone. Westman ('31) reports many large but mostly empty vessels in the uterine mucosa in rabbits after the administration of this hormone. Moreover, after experimental withdrawal of progestin blood cells appear in the lumina of the vessels and

immediately thereafter the size and number of open vessels decreases.

The regression in area of the vascular bed between the twenty-third and twenty-eighth day of pregnancy as reported herein is in agreement with the observations of Markee and French ('34), Barcroft, Herkel and Hill ('33) and Reynolds ('37a, b). Markee and French observe a decrease in color of transplants after the twentieth day, reaching a low point between the twenty-fifth to the thirtieth day. Barcroft, et al. find that the rate of blood flow is one third less between the twenty-fifth and twenty-seventh day than between the twentieth and twenty-fourth days. Reynolds ('37a), using Barcroft's data, calculates the percentage blood volume which changes each minute (the efficiency of the maternal circulation) in the uterus during various stages of pregnancy. He finds this increases slowly to the twenty-second day and then decreases markedly by the twenty-fifth day, and that this lowered efficiency is maintained for several days. Although the findings of these investigators may be changes of color, rate of blood flow, or per cent of blood volume changed each minute, they are all reported to appear at about the same time and the direction of change is the same in all of them, namely, toward a decrease in blood flow. The morphologic evidence submitted here likewise indicates a similar change.

In the last 3 days of pregnancy there is probably a reduced blood flow in spite of an extensive vascular bed filled with erythrocytes, but since the vascular bed makes up a large percentage of the volume of the endometrium it does explain Markee and French's observation that transplants show their most intense color during the 1 to 3 days preceding parturition, and reach a peak on the day of delivery. In intraocular transplants the color is a bluish or purplish red rather than a bright red. This has been observed many times in endometrial transplants both in the course of this study and by Markee and French. The endometrium in situ has a similar appearance when observed through a glass window in the abdominal wall (personal communication from Dr. Markee).

His observations that the red blood cells circulate very slowly (some may not move for a full minute) indicates that, in the transplants at least, the bluish color observed is due to a pronounced stasis. The great concentration of red blood cells at this time may mean that the edema always present at the time of parturition (Krichesky, '42) is caused by an escape of plasma from blood which is circulating so slowly.

It is surprising that it is possible to obtain information on vascular changes from fixed materials. On the death of the animal the disappearance of blood changes the color of the transplants from a deep pink to a grayish white before the tissue can be excised. Markee (unpublished) finds similar changes in color in both endometrial transplants and in the uterus in situ on induction of anesthesia, many of his observations being made through a glass window in the abdominal wall.

SUMMARY

1. During the first 4 days of pregnancy there is a marked hyperemia in the superficial region of the mucosa. From the seventh until the twenty-third day there is a gradual increase in the area of the vascular bed accompanied by a marked reduction in the number of blood cells in their lumina. From the twenty-third to the twenty-eighth day there is a marked reduction in the area of the vascular bed. Following this reduction, and continuing to the day of delivery the area of the vascular bed increases. At this time there is a great increase in the concentration of red blood cells suggesting stasis. After delivery the vascular bed diminishes until the non-pregnant condition is attained by the twelfth day post partum.

2. Numerous tissue spaces occur in the stroma 2 days prior to delivery until 2 days after delivery. Many of these, but not all, probably are due to contractions of the muscularis. Their origin is temporally related to the appearance of extensive edema and may indicate that the connective tissue is more friable at this time.

3. The changes in the endometrial vascular bed in the uterus and transplants reported herein adequately account for the findings on changes in color of endometrial transplants during pregnancy.

4. In spite of the shifting of blood out of the uterus and endometrial transplants on death of the animal, it is surprising that the sequence of vascular changes observed in fixed tissues is so completely compatible with direct observations on color changes and rate of blood flow in living endometrium.

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THE HUMAN STERNOCHONDRAL JOINTS

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ONE TEXT FIGURE AND THREE PLATES (TWENTY-FOUR FIGURES)

INTRODUCTION

The sternochondral joints, except for the first, are usually described as being freely movable and possessing a joint cavity. They are therefore classified as diarthrodial joints. A "tendency" to become synchondrodial has been noted, however, especially in the sixth and seventh joints. This process has been attributed to ageing.

A transverse intra-articular fibrocartilaginous ligament is described for the second sternochondral articulation when a cavity is present. This is reported to be of fairly constant occurrence, and to be found occasionally in the other joints. Sappey (1867) noticed that this ligament is located in the anterior part of the articulation and observed that sometimes it is vestigial, while at other times it completely fills the joint cavity. Fick ('04) found the ligament to be thinner behind than in front, and observed that it occasionally fails to reach the dorsal capsular wall, leaving a space by means of which the upper and lower parts of the cavity communicate.

Henle (1871) found the intra-articular ligament to be variable in position. Sometimes it occurs higher than the mid-plane of the joint cavity, sometimes lower, and at other times is totally lacking. Hyrtl (1882) considered the ligament to be a true prolongation of the fibrocartilage uniting the manubrium with the body of the sternum.

In order to observe the frequency of the presence of the intra-articular ligaments, Tschaussow (1891) studied the

sternochondral joints of eighty-nine cadavers. In the second joint they were absent in three bodies. In the third joint they were present eighteen times, in the fourth nine times, and in the seventh four times. No figures were given for their presence in the fifth and sixth joints.

Musgrove (1892-93) found the second joint divided into two cavities on the right side in eleven out of eighteen bodies. There were double cavities in the third joint five times, in the fourth twice and in the fifth twice. The sixth and seventh right joints showed either a single cavity or none at all. On the left side there were two cavities in the second joint ten times, in the third seven times, in the fourth twice and in the fifth once. No double cavities were observed in the sixth and seventh joints of the left side.

In regard to the presence of an intra-articular ligament in the second joint, Bryce's ('15) figure of about 60% agrees more closely with that of Musgrove than that of Tschaussow.

The investigations of Tschaussow have been quite widely accepted as far as the appearance of cavities is concerned. He claimed the appearance of whitish bands to be the first indication of future joints and asserted that cavities appear first at the union of the sternum with the third costal cartilages in a 5-month fetus. The cavities of the second articulation appear at a slightly later stage, and still later in the remaining joints. Contrasted with these observations are those of Pohlmann ('33) who claimed never to have seen cavities in embryonic sternochondral joints.

MATERIALS AND METHODS

The sternochondral joints of a series of twenty-two human embryos and fetuses,¹ ranging in age from 6 weeks to term, were studied in an attempt to determine the time of appearance of the cavities. All of this material had been preserved in 5% formalin. The four youngest specimens were washed,

¹ Four of the fetal specimens were obtained from Washington University School of Medicine, St. Louis, Missouri, during a year spent by the junior author in the Department of Anatomy there.

dehydrated, embedded in Tissuemat, serially sectioned in a transverse plane at 10 micra, and stained by the Mallory-Azan method. The sterna, including parts of the costal cartilages, were removed from the eighteen older fetuses and were then washed and decalcified in 5% nitric acid. Eight of these were dehydrated, embedded in Tissuemat and cut serially in a frontal plane at 12 micra. Alternate sections were mounted and stained by the Mallory-Azan method. The remaining ten sterna and costal cartilages were embedded in nitrocellulose and cut serially in a frontal plane at 50 micra. Every third, fifth or tenth section, depending on the size of the sternum, was stained with hematoxylin and eosin and mounted.

Studies of adult articulations were made on dissecting room material. The joints of ninety-six bodies, ranging in age from 33 to 92 years, were examined. The sterna, with the medial portions of the costal cartilages, were removed from the cadavers and sectioned in a frontal plane so as to bisect the joints. Breakage, or deviations from the line of sectioning, rendered a few articulations unfit for study. Examinations of the suitable joints were made to determine grossly the frequency of the presence of cavities, and of strands or ligaments crossing the cavities. Representative joints were removed from these specimens and decalcified in 5% nitric acid. They were then washed and dehydrated. Of these, approximately one-half were infiltrated with nitrocellulose dissolved in methyl benzoate and subsequently embedded in Tissuemat, cut serially in a frontal plane at 10 micra, and stained by the Mallory-Azan method. The remainder were embedded in nitrocellulose, cut serially in a frontal plane at 15 micra, and every tenth section was stained with hematoxylin and eosin.

OBSERVATIONS

Development (to term)

In all the embryos there were direct cartilaginous unions of the sternal bar with the costal cartilages (figs. 2 and 14). The future sternochondral joints were indicated at 3½ months,

however, when zones of demarcation were present in the pre-cartilage of these areas (figs. 5 and 15). The parts of the costal cartilages and the sternum adjacent to these demarcation zones were acidophilic. At this or slightly later stages the zones themselves had lost their cartilaginous nature and had progressively assumed the character of a loose connective tissue. After the cartilaginous character was definitely lost, the cells within the demarcation zones increased in size and became arranged in parallel rows between the fine bundles of collagenous fibers which passed transversely across the zones (figs. 3 and 17). The tissue thus formed bore some resemblance to early fibrocartilage.

Minute spaces appearing between the rows of cells and among the fibers which traversed these zones coalesced and thus became somewhat larger. This process was especially apparent in the central parts of the zones although the first definite joint cavities to appear were usually at the periphery (figs. 4 and 16). The time interval between the appearance of the demarcation zones and the formation of cavities varied considerably. In the second joint, cavities were usually formed during the third month, shortly after the demarcation zones were first indicated. If not, cavities in this and other joints usually appeared at later times during fetal life.

In one $3\frac{1}{2}$ -month-old fetus cavities were present bilaterally in the second sternochondral joint and were separated into upper and lower parts by an intra-articular ligament which was continuous with the tissue separating the manubrium from the body of the sternum (figs. 6 and 18). In the other specimens of this age cavities were present only in the upper halves of the joints. Except for one full-term fetus in which they were absent in all of the joints, there were cavities in the second on one or both sides in all fetuses from $3\frac{1}{2}$ months to term.

Cavities appeared in some third joints at 4 months. In two out of seven specimens they were unilateral. Bilateral cavities were not present in the third joint until term, and then in only one specimen out of four at this age.

Cavities were found in neither the fourth or fifth joints except in one full-term fetus in which they were present bilaterally in both joints. Cavities were found in the sixth and seventh joints only twice; once in a fetus 6 months old and in another at term. These were all bilateral. In one fetus at term, cavities were found in every sternochondral joint.

Adult joints

Gross appearance. Variations from the usual textbook descriptions of the second sternochondral articulation were frequently encountered. Only 30% of ninety-two right second joints had cavities both above and below the intra-articular ligament. Eight per cent showed single cavities, due to the absence of the ligament while 11% had no cavities. In 51% either the cavities were crossed by strands or one cavity was absent. When one cavity was absent, it was almost always the upper.

On the left side 42% of the second joints had complete upper and lower cavities. Five per cent had complete cavities, but lacked intra-articular ligaments. Those without cavities amounted to 10% of the total examined. Forty-three per cent exhibited strands or the complete absence of an upper or lower cavity. As on the right side it was the upper cavity which was usually absent.

The percentages of all the articulations which had cavities, no cavities or partial cavities are shown in table 1.

The joints other than the second also varied considerably in gross appearance when they were examined in frontal sections. The occurrence of strands which crossed some of the cavities from the sternal articular facets to the costal cartilages was most striking. These differed in size, number and direction. They varied from exceedingly thin strands to those of a thickness comparable to that of the intra-articular ligament of the second joint. They usually crossed the cavity obliquely, but sometimes transversely. Partial or complete obliteration of the joint cavity was sometimes accom-

plished by a solid mass of tissue similar in appearance to these strands.

Other irregularities were sometimes present. There were occasionally small knobs or protuberances on one articular facet which fit into corresponding depressions on the opposed surface. Where cavities were partially or completely present, the articular cartilages had a dense appearance, were whitish in color and lacked the usual translucence of hyaline cartilage. The line of demarcation between the hyaline cartilage of the

TABLE 1
Incidences of cavities and strands in sternochondral joints from the dissecting room.

	FREQUENCIES IN PER CENT (NEAREST ROUND NUMBER)			
	Number of cases	No cavities	Uninterrupted cavities	Cavities crossed by strands
Second right	92	11	38	51
Second left	89	10	47	43
Third right	95	19	40	41
Third left	95	23	35	42
Fourth right	94	23	43	34
Fourth left	93	40	35	25
Fifth right	96	25	41	34
Fifth left	94	34	29	37
Sixth right	94	52	26	22
Sixth left	94	59	20	21
Seventh right	88	59	17	24
Seventh left	86	71	15	14

costal cartilage and this tissue which lined its articular end was ordinarily quite abrupt and easily visible to the naked eye. Similarly, on the sternal side the demarcation between bone and articular cartilage was sharp.

The presence of characteristic intra-articular ligaments in joints other than the second was rare. In a very few instances a band crossing the middle of the third articulation resembled somewhat the ligament of the second joint, but in joints below the third no such band was observed.

Microscopic appearance. Fibrocartilage was found in all the sternochondral joints examined, whether or not cavities were present, and constituted the tissue intervening between

the costal cartilages and the sternal facets. In the second joint the fibrocartilage forming the articular surfaces was directly continuous with the cartilage separating the manubrium and the body of the sternum (figs. 8 and 20). When cavities were present in any of the joints they usually divided the fibrocartilage into almost equal parts and ordinarily extended to the capsule above and below.

On the sternal side of all the sternochondral joints there was a layer of hyaline cartilage which separated the articular fibrocartilage from bone. This layer was sometimes extremely thin or even deficient in places (fig. 21). In some cases small areas were found in which the compact bone of the sternum was lacking and thus the bone marrow lay adjacent to either hyaline or fibrocartilage. Occasionally blood vessels were seen passing through these areas.

A sternal articular fibrocartilage was often comparatively thick; if it was, the fibrocartilage on the opposite side of the cavity was usually thin (fig. 9). That part of the fibrocartilage adjacent to the cavity was acellular, amorphous and quite acidophilic (fig. 22). This was true also of the free borders of the strands which crossed the cavities (fig. 23).

On the costal side of the joint cavity the transition between fibrocartilage and costal cartilage was in some cases quite abrupt, and in others rather gradual with the collagenous fibers of the fibrocartilage continuing for a short distance into the hyaline cartilage. Both articular fibrocartilages could usually be traced into the perichondrium or periosteum at the upper or lower parts of the joint. In a few cases, however, there was a direct continuity of these cartilages, either above or below the cavity (fig. 10). They were rarely continuous at both extremities.

The bands crossing the joint cavities were extremely variable. They sometimes occupied one-half or more of the cavity, while at other times they were sufficiently small to be almost unrecognizable under low magnifications of the microscope. While most of them crossed the cavities in an oblique direction from superior to inferior and from anterior to pos-

terior, there were occasionally those which were transverse or nearly so. In all the sections studied the strands were composed of fibrocartilage (figs. 11 and 23).

In joints which had no cavities, the fibrocartilage covering the sternal facet was directly continuous with that covering the costal cartilage, with no line of differentiation between the two (figs. 12, 13, 24 and 25). There was no constant arrangement of the cells and fibers of the fibrocartilage. At times they were arranged irregularly (fig. 25). More commonly, however, the cells and fibers had a tendency toward a more or less longitudinal arrangement at the upper and lower limits of the joint, while in its middle part columns of cartilage cells ran in a transverse direction and were separated by collagenous fibers passing in the same direction (fig. 24). A less dense arrangement of both cells and fibers was noticed as the center of the joint was approached.

In the right third joint of one specimen a peculiar extension of the capsule was noted. Dividing the joint cavity into two parts, in much the same manner as an intra-articular disc, was a band of tissue continuous above and below with the capsule. This band at the periphery had a synovial covering and contained blood vessels. Centrally it became amorphous, acellular and acidophilic.

In a few joints loose connective tissue with numerous small blood vessels was located at the superior and inferior extremities of the joint, and in one this very vascular tissue completely obliterated the cavity.

Ossification of the sternal extremities of the costal cartilages did not involve the fibrocartilage. It was also observed that even in old age the body of the sternum may not be completely ossified. Areas adjacent to the sternochondral joints now and then contained small patches of hyaline cartilage completely surrounded by bone.

DISCUSSION

The appearance of cavities in the sternochondral articulations as shown by this study is subject to considerable varia-

tion. When the investigations are confined to embryonic and fetal material, the order of appearance of cavities cannot be learned for all joints since some cavities may appear after birth. It seems safe to state, however, that the first cavities to appear are those of the second articulation. A cavity in the third joint usually follows the second at a slightly later stage, although in one 6-month fetus bilateral cavities were present in both sixth and seventh joints, while the cavity of the third joint was still unilateral.

The initiation of fetal respiratory movements as postulated by some investigators to be responsible for the formation of cavities does not seem to be a reasonable explanation, inasmuch as cavities appeared in some fetuses as early as the third month, while in others they were still not present at the time of birth. The order of appearance of cavities as given by Tschaussow was not confirmed by this study, nor do these results agree with those of Pohlmann.

With the lack of specimens from birth to 33 years of age, the question arises as to whether joints which showed no cavities in the adult ever developed them, since the percentage of fetuses showing an absence of cavities was comparable to that of the adults in which the cavities were absent.

The manner in which the fibrocartilage appears in the adult joint gives rise to speculation. It was stated earlier that the tissue which lay in the demarcation zones had some resemblance to early fibrocartilage. It may be postulated that this tissue never disappeared, but remained to become the fibrocartilage in the adult joint. This does not, however, account for the fact that after the appearance of cavities in the fetal joints, the tissue of the demarcation zones disappeared and there remained only the articular hyaline cartilages of the sternum and costal cartilages (figs. 7 and 19). Yet in the adult all the sternochondral joints examined contained fibrocartilage.

It is possible that the fibrocartilage may arise through a transformation of the hyaline cartilage. The small amount, or lack of hyaline cartilage on the sternal articular facet

seems to suggest this. This provides no explanation, however, for the manner in which the fibrocartilage crosses the joint cavity.

It is also possible that the cartilage may arise from the capsule, or even from the sternal bone marrow, since the latter may be in direct communication with the fibrocartilage. Moreover, the time of appearance of adult fibrocartilage here is unknown. It is to be hoped that specimens of the ages not now available will aid in solving these problems.

It is known that fibrocartilage may appear in joints which are subject to unusual mechanical stresses and there is the possibility that stresses sufficient to bring about this change occur in the sternochondral articulations. Although they carry no weight, the repetition of movement and the movements in many directions bring about conditions to which other joints are not subjected.

It is apparent from figure 1 that the percentage of joints lacking cavities increased from the second to the seventh joints. Conversely the percentage of joints showing complete cavities and those having strands tended to decrease from the second to the seventh. These findings are impossible to correlate with an assumption that cavities are necessary for movement since it is well known that the lower ribs are subject to greater ranges of motion during respiration than the upper.

From these observations it seems that the use of the term intra-articular ligament is not justified except when it is used in connection with the second joint. What may appear grossly to be a band or ligament often proves to be a number of separate strands, and these strands are not necessarily parallel to each other.

There is a strong likelihood that strands running transversely across the joint cavity would tend to restrict motion. The usual direction of the strands, however, is oblique, and they may run in such a way as to make considerable movement possible, although it is to be admitted that the presence of

strands suggests a smaller amount of movement than does a complete cavity.

The absence of cavities in the sternochondral joints does not imply lack of motion. They are absent much too frequently to think that movement was lacking or even greatly restricted. A certain amount of compression of the fibrocartilage in such joints necessarily occurs.

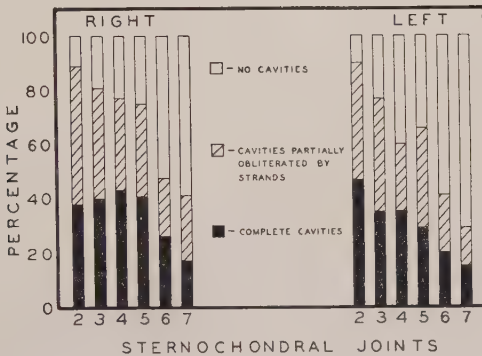


Fig. 1 Graphs illustrating the incidence of joints with complete cavities, with no cavities and with cavities crossed by strands.

The question of age in connection with the presence or absence of cavities was considered, and results of the findings were tabulated according to the age of the cadaver. No correlation was observed. The joints in younger bodies were as apt to lack cavities as those of the older. On the other hand cavities were as frequent in the older specimens as in the younger ones. It should be understood that the factor of age is not necessarily ruled out by these findings. It is probable that joints from a series of bodies varying in age from birth to 33 years will show some progressive changes.

SUMMARY

1. The times of appearance of cavities in the sternochondral joints are subject to wide variation. They were present

in some joints in the 3½-month fetus. On the other hand a fetus at term showed cavities in none of the sternochondral joints.

2. The cavities of the second joint were the first to make their appearance. The cavities of the third appeared shortly afterwards, at least unilaterally. Insufficient material precludes establishing with certainty the order of appearance of the remaining cavities. Present indications are that those of the sixth and seventh joints follow the third in appearance, while those of the fourth and fifth appear last.

3. The adult articulations showed a decreasing percentage having complete cavities in passing from the second to the seventh joint. The percentage having strands across the cavity also decreased from the second to the seventh, while the proportion exhibiting complete lack of cavities increased in this progression.

4. The intra-articular ligament of the second joint was absent in 8% on the right side, 5% on the left. If either upper or lower cavity of this joint was absent, it was almost always the upper.

5. All sternochondral joints contained fibrocartilage between the sternal articular facets and the costal cartilages. Any cavities which were present divided it into two articular fibrocartilages.

6. Strands crossing the cavities were always composed of fibrocartilage. These strands usually passed obliquely, rarely transversely across the cavities. They varied in size and number.

7. Age does not seem to be a factor in determining the presence or absence of joint cavities in subjects from 33 to 92 years.

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PLATE 1

EXPLANATION OF FIGURES

2 Cross section of a 2-month-old human embryo in which the sternal bars and some costal cartilages are cut in the frontal plane and are directly continuous with each other. $\times 2\frac{1}{2}$.

3 Frontal section through a fourth sternochondral junction of a full term fetus. A demarcation zone is evident. Note numerous blood vessels in sternum and costal cartilage. $\times 5$.

4 Frontal section through sternum and costal cartilages of a 4-month fetus. That part of the second sternochondral junction in the outlined area is illustrated in figure 16. $\times 5$.

5 Frontal section through the sternum and costal cartilages of a $3\frac{1}{2}$ month fetus. The demarcation zone of the third sternochondral junction is illustrated in figure 15. $\times 5$.

6 Frontal section through the second sternochondral junctions of a $3\frac{1}{2}$ month fetus. The junction which is outlined is illustrated in figure 18. $\times 5$.

7 Frontal section through the second sternochondral junctions of a full term fetus. The cavity indicated by the arrow is illustrated in figure 19. $\times 5$.

8 Frontal section of an adult left second sternochondral joint in which upper and lower cavities are separated by an intra-articular ligament. Figure 20 illustrates the area outlined. $\times 5$.

9 Frontal section through an adult right third sternochondral joint in which there is a slit-like cavity and a thick pad of fibrocartilage on the sternal side. See figure 21 for area outlined. $\times 5$.

10 Frontal section of an adult left third sternochondral joint. An irregular cavity bounds a protuberance on the costal side. There are a few fibrocartilaginous strands at the periphery of the cavity. The articular fibrocartilages are continuous at the lower part of the cavity. The outlined area is illustrated in figure 22. $\times 5$.

11 Frontal section of an adult left fifth sternochondral joint. A large oblique band crossing the cavity is indicated by an arrow and is also illustrated in figure 23. $\times 5$.

12 Frontal section through a completely closed adult right fourth joint. $\times 5$.

13 Frontal section of a completely closed adult left second joint. The fibrocartilage occluding the cavity is directly continuous with that separating the manubrium from the body of the sternum. $\times 5$.

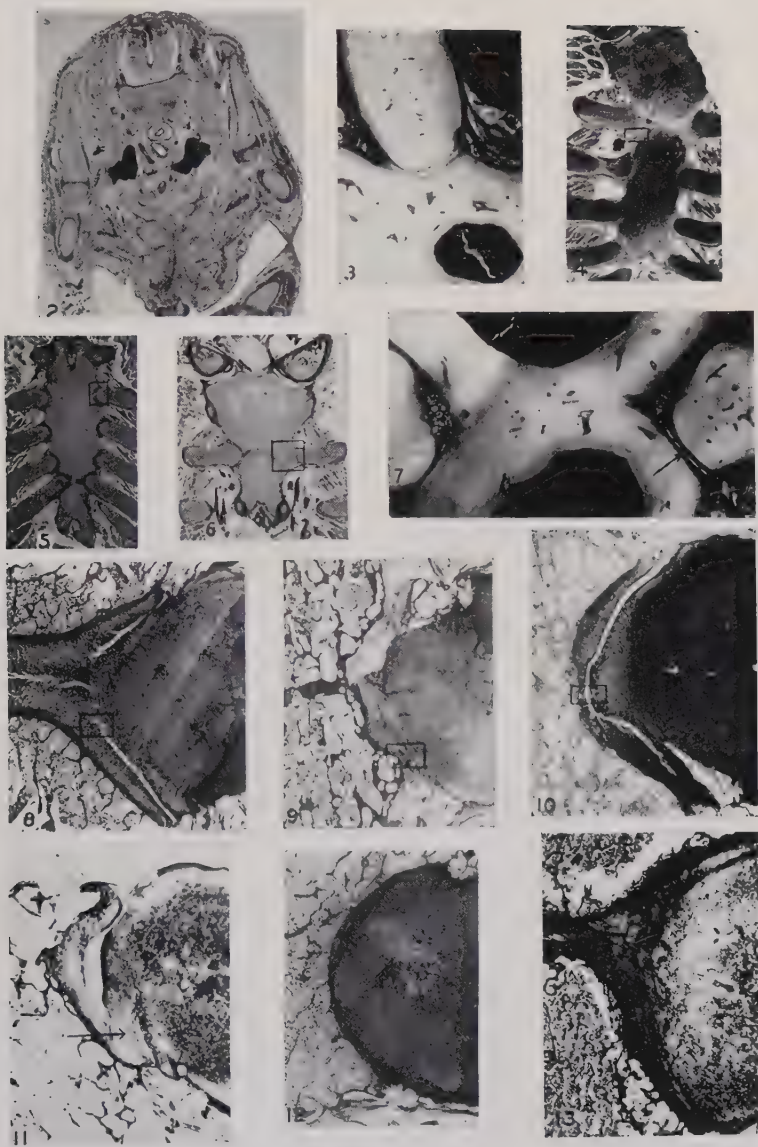


PLATE 2

EXPLANATION OF FIGURES

- 14 Frontal section through three sternochondral junctions of figure 2. $\times 60$.
- 15 Frontal section through an early demarcation zone of a third sternochondral junction of figure 5. $\times 60$.
- 16 Frontal section through the second sternochondral junction of figure 4. Early cavity formation indicated at periphery of junction. $\times 60$.
- 17 Frontal section through the third sternochondral junction of figure 3. Transverse striations of an advanced demarcation zone. $\times 60$.
- 18 Upper and lower cavities in a second sternochondral junction of figure 6. $\times 60$.
- 19 Well advanced cavity with articular hyaline cartilage of second sternochondral junction of figure 7. $\times 60$.

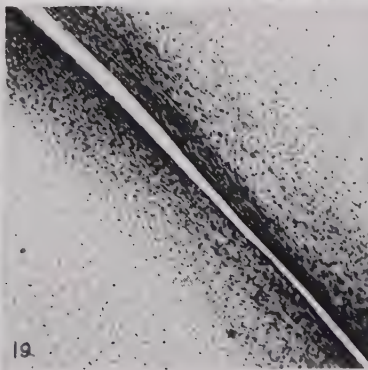
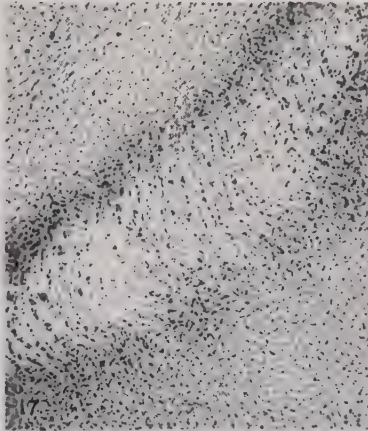
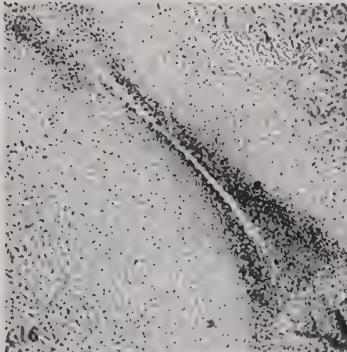
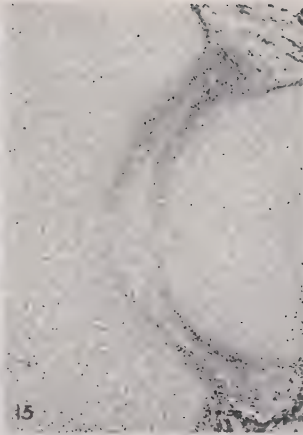
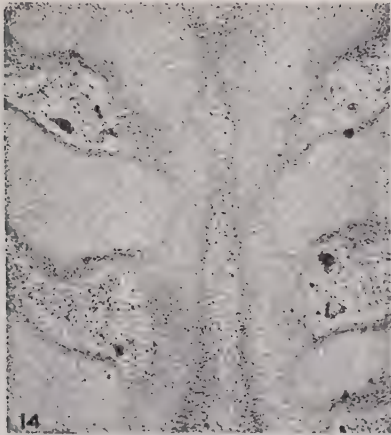


PLATE 3

EXPLANATION OF FIGURES

20 Cavity, articular fibrocartilage and a fibrocartilaginous strand of second sternochondral junction of figure 8. $\times 60$.

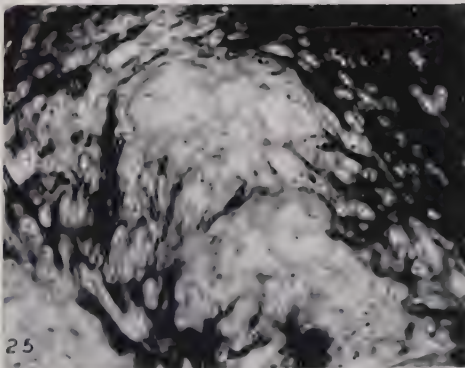
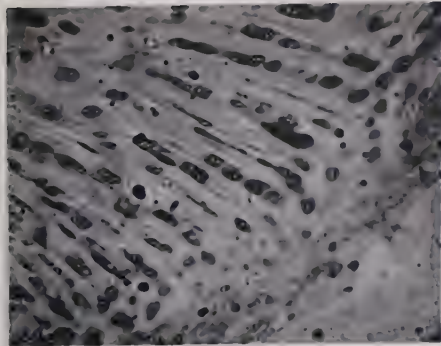
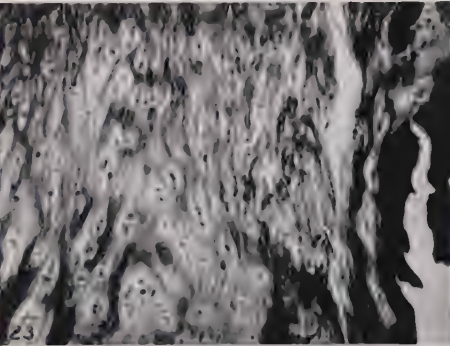
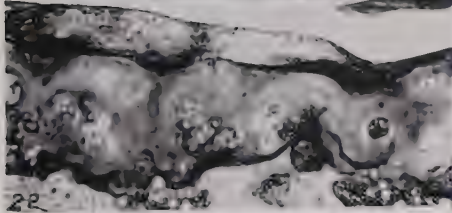
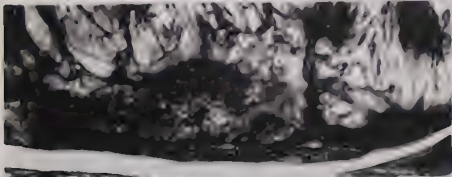
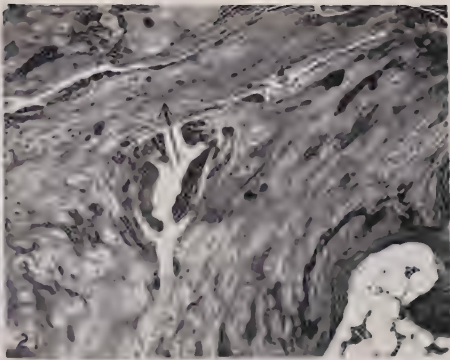
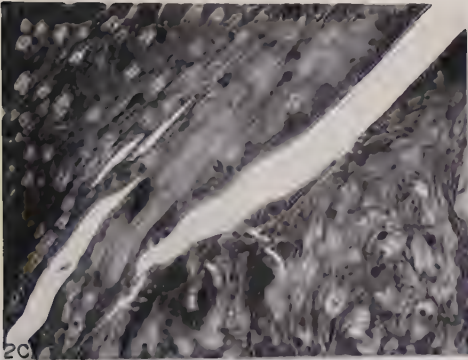
21 Heavy fibrocartilaginous strand (arrow) crossing the cavity of the third joint illustrated in figure 9. Note the fibrocartilage in contact with compacta of sternum in lower right corner. $\times 60$.

22 Articular fibrocartilages and a small fibrocartilaginous strand of the left third sternochondral joint shown in figure 10. Note amorphous character of free border of articular fibrocartilages. $\times 60$.

23 Fibrocartilaginous band crossing the cavity of the fifth joint illustrated in figure 11. Note again the amorphous free border of the fibrocartilage. $\times 60$.

24 Fibrocartilage obliterating the cavity of the right fourth joint illustrated in figure 12. Note the transverse arrangement of the cells and fibers near the central part of the joint, and the longitudinal arrangement toward the periphery in the upper right corner. $\times 60$.

25 Fibrocartilage obliterating the cavity of the left second joint shown in figure 13. Here the cells and fibers are arranged in a very irregular manner. $\times 60$.



COMBINED ANTERIOR AND POSTERIOR SPINA BIFIDA IN A LIVING NEONATAL HUMAN FEMALE

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TEN FIGURES

Human specimens with a cleft vertebral column or combined anterior and posterior spina bifida are rare. Cruveilhier (1824) described the first case, but Gruber ('26) and Feller and Sternberg ('29) could only collect thirty-one cases between them. To date only thirty-seven cases appear to have been described, and of these but two — Adelmann ('20), Bell ('23) — deal directly with the subject in the English language.

The present case falls into group 2 of Feller and Sternberg's classification. This group encompasses those cases in which some part of the intestinal tract passes dorsally through a cleft vertebral column and spinal cord to present itself on the back. The only two previously described in this group are those of Lucksch ('03) and Adelmann ('20).

Lucksch's specimen (human) had an upper dorsal defect in contrast to the lumbo-sacral defect of this case, which appears unique in human teratology since Adelmann's specimen, although closely corresponding, was a calf.

Combined spina bifida is an extreme in axial growth phenomena and may therefore elucidate some lesser abnormalities of the spine. For instance the Clinical Society of London (1885), Cleland (1889) and Greig ('29) have hinted at the possibility of a relationship between a cleft vertebral column (combined spina bifida) and the bony processes, attended

by varying degrees of spinal cord division, which project into the spinal canal in certain cases of posterior spina bifida (Humphry 1886, Ballantyne '04, Hamby '36).

CLINICAL HISTORY

The subject of the study, L. F. R., a female White infant 2 days old, was admitted to hospital because of a rounded red mass of mucous membrane that projected from the lumbar region of the back (fig. 1).



Fig. 1 Schema showing communication of hernial loop with fistula.

This mass was continuous superiorly and laterally with the adjacent skin. Inferiorly it overhung a small fistula which exuded meconium. A movement suggestive of a peristaltic wave was seen sweeping over the mass and next day undigested curd and meconium fouled the protective dressing.

A radiograph revealed a cleft lumbar spine and a catheter introduced into the fistula emerged at the anus. Radiography with Abrodil in the catheter indicated that the bowel communicated with the surface through the cleft spine.

During the second month in hospital the mass steadily grew larger, feces eventually being passed both per fistulam et

anum. The child gradually lost weight and died of sepsis and inanition early in its fifth month.

After death the body was placed in alcohol and passed into the hands of Colonel W. F. Harvey, Superintendent of the Royal College of Physicians Laboratory, Edinburgh, who generously placed the specimen at the author's disposal.

ANATOMICAL OBSERVATIONS

The body was well formed. The various measurements of the body and dorsal mucous mass or pad are given in table 1. The skin about the pad was mottled and elevated 20 mm. above the surrounding skin level. Microscopic section of the mucous pad showed its structure to be characteristic of large intestine.

Thorax and abdomen

The thoracic contents and diaphragm were normal. On opening the abdomen no transverse colon was found. Instead, a hernial loop of large bowel passed back into a large, low lying, median fossa lined with peritoneum (fig. 2). Composed of entering and returning limbs, the loop communicated with the fistula on the back (fig. 1).

The stomach was J-shaped. The proximal part of the duodenum adhered to the back wall; the rest was attached by a mesoduodenum that contained the pancreatic head, and lay anterior to the superior mesenteric artery.

The small intestine was short (table 1) and entered the right side of the caecum which lay in the left iliac fossa. A continuous "common mesentery" composed of mesoduodenum and mesentery thus passed from the fixed proximal part of the duodenum down to the left iliac fossa. It lay both above and to the left of the midline fossa.

The entering limb of large bowel ("ascending colon") entered the midline fossa above the returning limb ("descending colon"). The peritoneum was reflected on to the hernial limbs and formed culs-de-sac on either side of them. Re-

moval of the peritoneum revealed that the midline fossa was bounded by the hemivertebrae or halves of the cleft vertebral column. The cleft extended from the first lumbar to the second sacral vertebra inclusive (fig. 4).

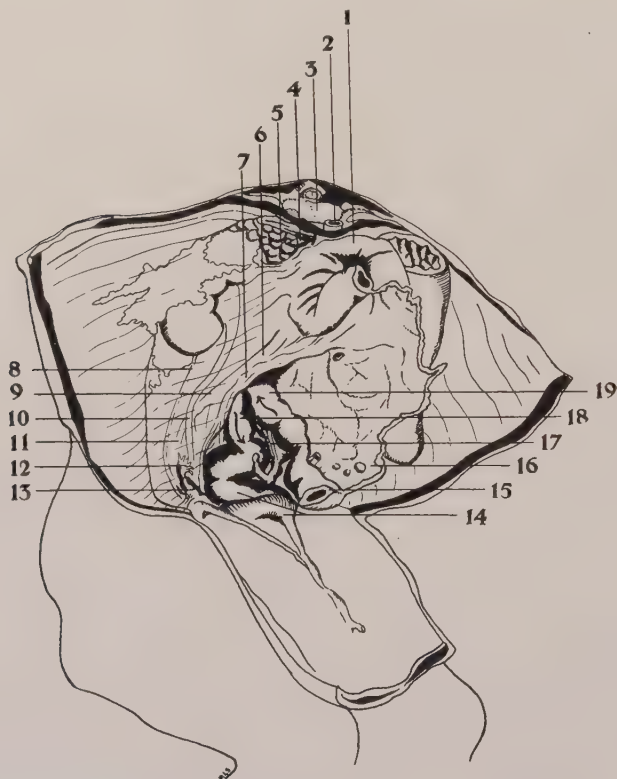


Fig. 2 The midline peritoneal fossa and its hernial loop.

- | | |
|---|--------------------------|
| 1. Duodenum | 10. Ureter |
| 2. Aorta | 11. Right External Iliac |
| 3. 11th Thoracic Vertebra | 12. Fimbriae |
| 4. Inferior Vena Cava | 13. Right Horn of Uterus |
| 5. Suprarenal | 14. Rectum and Bladder |
| 6. Aorta | 15. Ileum |
| 7. Vessels replacing Inferior Mesenteric in crescentic peritoneal fold skirting fossa | 16. Mesentery |
| 8. Right Ovarian Vessels | 17. Caecum |
| 9. Right Common Iliac | 18. Returning Limb |
| | 19. Entering Limb |

Genito-urinary system

Figure 3 shows the disposition of the renal vessels, ureters and kidneys about the spinal cleft. The left kidney was abnormal. The body of the pancreas lay transversely just above the cleft. The suprarenals, bladder and vagina were normal; the uterus was bicornuate.

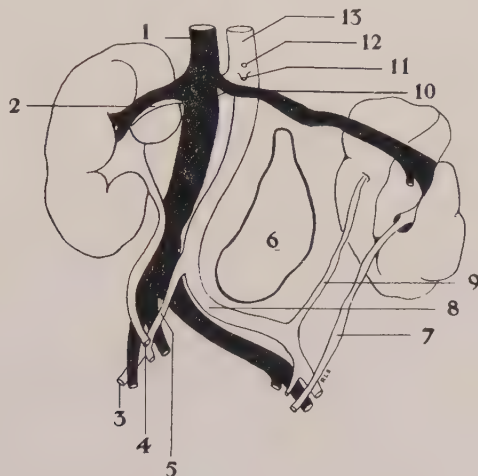


Fig. 3 The relationship of the abdominal vessels and renal structures to the spinal cleft.

- | | |
|---------------------------|-------------------------|
| 1. Inferior Vena Cava | 8. Left Common Iliac A. |
| 2. Right Renal V & A. | 9. Left Renal A. |
| 3. Right External Iliac A | 10. Left Renal V. |
| 4. Right Ureter | 11. Sup. Mesenteric A. |
| 5. Right Common Iliac A. | 12. Coeliac A. |
| 6. Spinal Cleft | 13. Aorta |
| 7. Left Ureter | |

Abdominal vessels

Figure 3 also shows the general arrangement of the abdominal vessels about the cleft. The lower end of the aorta inclined right to lie in front of the right lumbar hemi-vertebrae. The superior mesenteric artery entered the common mesentery and supplied the pancreas, small intestine, caecum and "entering limb" of large bowel. It also supplied the "returning limb" and rectum by vessels that ran in a

crescentic peritoneal fold on the right side of the midline fossa. There were no inferior mesenteric vessels.

Vertebral column

The vertebral column and its cleft are shown in figure 4. It was normal down to the seventh thoracic vertebra. The

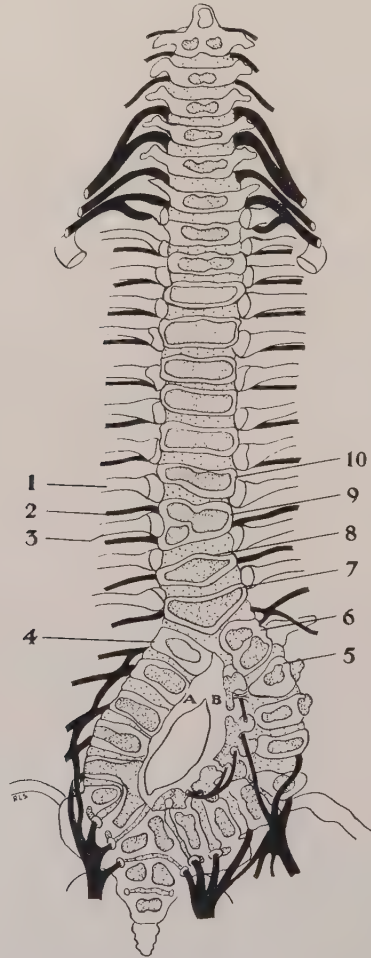


Fig. 4 The vertebral column and its cleft from in front. Points A and B overlie the dural sheaths of the right and left divisions of the spinal cord.

- | | |
|-----------------------------------|-------------------------------|
| 1. 9th Right Rib | 6. Intercalated Hemi-vertebra |
| 2. 9th Right Thoracic Nerve | 7. 12th Thoracic Vertebra |
| 3. 10th Right Rib | 8. 11th Thoracic Vertebra |
| 4. 1st Right Lumbar Hemi-vertebra | 9. Composite Piece |
| 5. 1st Left Lumbar Hemi-vertebra | 10. 8th Thoracic Vertebra |

eighth thoracic lay obliquely. Below it was a composite piece regarded as the ninth, and right half of a deficient tenth. A diamond-shaped eleventh and triangular twelfth followed.

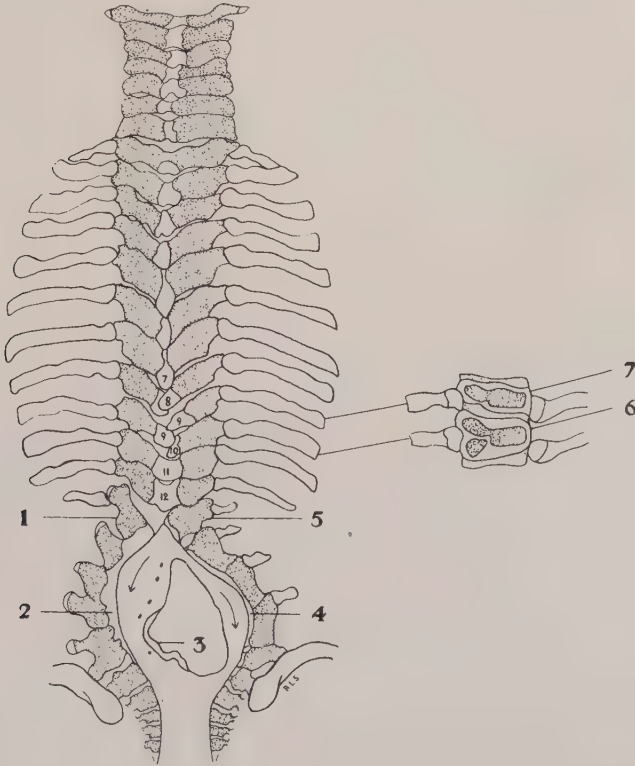


Fig. 5 The vertebral arches. Arrows indicate right and left halves of vertebral canal.

- | | |
|--|--|
| 1. Half arch of Intercalated Hemi-vertebra | 5. Half Arch of 1st Right Lumbar Hemi-vertebra |
| 2. Left Laminar Ridge | 6. Composite Piece |
| 3. Accessory Half Arch Ridge. | 7. 8th Thoracic Vertebra |
| 4. Right Laminar Ridge | |

The hemivertebrae flanking the cleft. On the right lay the right halves of the lumbar and upper two sacral vertebrae. These hemivertebrae, a laminar ridge formed by their fused half arches, and the right division of the spinal cord made up the right aspect of the cleft (figs. 4, 5).

On the left lay an intercalated hemivertebra and below it five lumbar and two sacral hemivertebrae. Their half arches fused posteriorly forming a laminar ridge. All except two (first and fourth L. Hemiv.) also had "accessory half arches" on their cleft aspect. These formed another ridge (fig. 4 and fig. 5, no. 3).

The "accessory half arch ridge," hemivertebrae, laminar ridge of true half arches, and left division of the spinal cord made up the left aspect of the cleft. The dimensions of the cleft are given in table 1.

TABLE 1
Dimensions

<i>Body lengths</i>	<i>Renal levels</i>
Crown rump 32.5 cm.	Rt. kidney . Th. 11 to 4th Rt. L. Hemi. V.
Over-all 47.5 cm.	Lft. kidney ... 1st to 5th Lft. L. Hemi. V.
<i>Mucous pad</i>	<i>Vascular levels</i>
Max. diameter 56 mm.	Aortic bifurcation 4th Rt. L. Hemi. V.
Max. thickness 25 mm.	Coeliac level 12th Th. V.
<i>Fistula</i>	Sup. mesenteric level 12th Th. V.
Max. diameter 4.5 mm.	Origin inf. vena cava .. 5th Rt. L. Hemi. V.
<i>Body weight</i>	<i>Dimensions of cleft</i>
On admission 5 lbs., 1 oz.	Max. median diameter 26 mm.
<i>Small intestine</i>	Max. oblique diameter 37 mm.
Pyloroduodenal to	Max. transverse diameter 25 mm.
Ileocecal junction .. 137.5 cm.	

The third sacral vertebra formed the lower end of the cleft. A bony spur on it completed the lower end of the accessory half arch ridge.

The vertebral arches. These and the laminar ridges are shown in figure 5. Fibrous tissue roofed the "open" sacral canal, and the two divisions of the spinal cord descending beneath the laminar ridges reunited beneath it. The ridges were responsible for the elevated skin about the dorsal mucous pad.

The vertebral formula. Computed from the right side this was C7, Th 12, L5, S5, C4. Serially considered it was the same on the left, the intercalated piece compensating for the in-

complete tenth thoracic. Actually the formula for the left appeared to be C7, Th 1-9, 11-13, L5, S5, C4.

The curvatures of the column. The cervical column was straight. The thoracic, hemivertebral and sacral regions were concave ventrally.

Intervertebral discs. To determine whether or not there had been a disturbance of the notochord, intervertebral discs were removed from above, below, and either side of the vertebral cleft. All possessed a nucleus pulposus. The nuclei pulposi between the right hemivertebrae were larger than those between the left.

The spinal cord and nerves

The spinal cord was normal to the seventh thoracic vertebra. Below that level both the spinal cord and its central canal increased in diameter. The cord bifurcated at the upper end of the vertebral cleft. The resultant divisions were unequal, the left being the larger; in both the central canal was dilated, reducing each to a thin wall of nerve tissue. The right division gave off a single laterally directed set of nerves, the left division both a lateral and medial set (fig. 6).

Each division descended in its half of the vertebral canal (see arrows fig. 5) and was related medially to the hernial loop and peritoneal culs-de-sac. Below the cleft the divisions and their dural sheaths reunited and the conus medullaris ended at the coccyx.

The spinal nerves down to the eighth thoracic were normal. The ninth left thoracic corresponded to the right ninth and tenth, the serially tenth and eleventh left thoracic being deemed the eleventh and twelfth since their cord attachments were opposite the right eleventh and twelfth.

The nerves on the right of the vertebral cleft were orderly, emerging below the vertebra of the corresponding number. On the left the nerves emerged in two sets, one on either side of the left half column, although both sets arose from the lateral aspect of the left division of the spinal cord. The lat-

eral set consisted of lower lumbar and sacral nerves. The medial set were small nerves whose roots embraced the left division as they passed to emerge through the accessory half arch ridge on the cleft. Certain of the medial set appeared to be accessory lumbar and sacral nerves (fig. 6, AL3, AL5, AS1.); they formed a looped plexus which lost itself in the

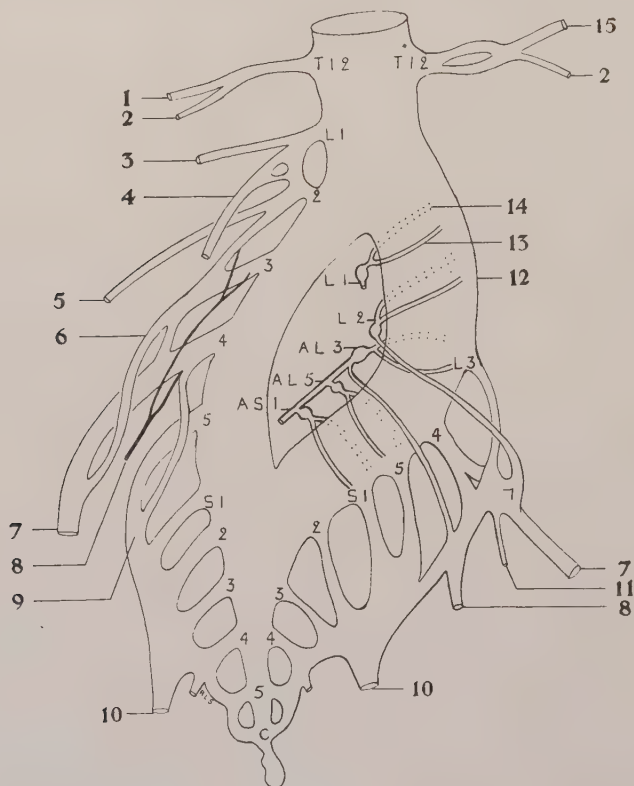


Fig. 6 Schema of the spinal cord in the region of the cleft. Note the two sets of nerves arising from the left division of the spinal cord.

- | | |
|-------------------------------|----------------------------------|
| 1. 12th Thoracic Nerve | 9. Lumbosacral Trunk |
| 2. Iliohypogastric | 10. Sciatic |
| 3. Ilioinguinal | 11. Accessory Obturator |
| 4. Genitofemoral | 12. Left Division of Spinal Cord |
| 5. Lateral Cutaneous of Thigh | 13. Anterior Root |
| 6. Accessory Anterior Crural | 14. Posterior Root |
| 7. Femoral | 15. Serially 11th Thoracic Nerve |
| 8. Obturator | |

tissue about the hernial loop. As will be seen from figure 6 the area between T12 and L3 was devoid of any nerve, but compensated by L1 and L2 appearing medial to the left hemivertebrae.

DISCUSSION

Nomenclature

Such a deformity may conveniently be termed a vertebral cleft (cf. German "Wirbelspalte"). Feller and Sternberg designate such cases "vordere Wirbelsäulenspalte (Rhachischisis anterior)," but more commonly "Wirbelkörperspalte," and imply an arch defect without specifying it. Korff ('37) speaks of "vollständiger Wirbelspalte (Rhachischisis anterior and posterior)."

The term anterior spina bifida has been used to denote a vertebral body and arch defect. Actually it should only be employed when the vertebral body alone is affected. When there is a concomitant posterior (arch) defect, the case should be spoken of as one of anterior and posterior spina bifida, or combined spina bifida.

Bell ('23) stated that true anterior spina bifida was only found in the sacral region, a posterior spina bifida usually accompanying it in the cervical and thoracic regions.

Anterior spina bifida should therefore be considered under two headings — (a) (True) Anterior Spina Bifida, and (b) Anterior and Posterior or Combined Spina Bifida. This subdivision is supported by the general features of the two types. Type (a) is usually confined to the sacrum. Characterized by anterior meningocele, it is, although rare, a well-known clinical entity which often permits the subject to attain maturity. Type (b) may involve any region of the vertebral column, but is most frequent in the cervical and thoracic regions. Often attended by marked alimentary and neural abnormalities it usually, but not invariably, results in pre- or neonatal death.

Classification

The present case falls within the category of type (b) or combined spina bifida. A survey of the records warrants certain general statements concerning this type. Involving any region of the vertebral column, and often attended by a cranial defect, it is characterized by a more or less extensive cleft of the vertebral bodies and arches. When the cleft is gross the vertebral halves arch laterally like a lyre, but otherwise show no tendency to diverge.

The alimentary tract is usually closely related to the vertebral defect. In some specimens, part of it passes through the cleft and either protrudes or opens on the back. In others, a strand¹ or diverticulum, or both, connects it with the spinal contents. In other words, the alimentary tract, depending on whether there is a single or cleft spinal cord (or medullary plate), connects with or passes through the spinal contents. The esophagus, stomach, and small intestine are the parts most commonly connected with such clefts (fig. 10). Sometimes alimentary adnexa such as the spleen are involved.

Not infrequently there is a left-sided diaphragmatic defect. The stomach and intestine are then found displaced above the diaphragm. Such findings more commonly attend a cleft of the upper part of the column; no doubt an early established "neurenteric" connection interferes with the growth of the alimentary primordium and by retaining its derivatives at an abnormal level also interferes with diaphragmatic development. Budde's ('11) sketches illustrate this and show how growth may elongate such a connection.

Various attempts at classification have been made. Bell ('23) in a review of cases showing an anterior and posterior spinal defect, employed a supra- and infra-diaphragmatic classification. Feller and Sternberg based their classification on the relationship of the axial body structures (central ner-

¹ Such a strand or diverticulum usually consists of nerve tissue at its "neural end" and smooth muscle at its "visceral end." For convenience they will be referred to as "neurenteric" connections.

vous system, vertebral column, alimentary tract) to one another. Their groups may be summarized as follows (fig. 7).

Group 1. The alimentary tract traverses a vertebral cleft and opens on the center of a modified medullary plate or area medullo-vasculosa. Representative cases² — Gruber (ii), Lehmann-Facijs, Lucksch (iii), Morel and Gross, Rembe, Schlippe, Stoltzenberg.

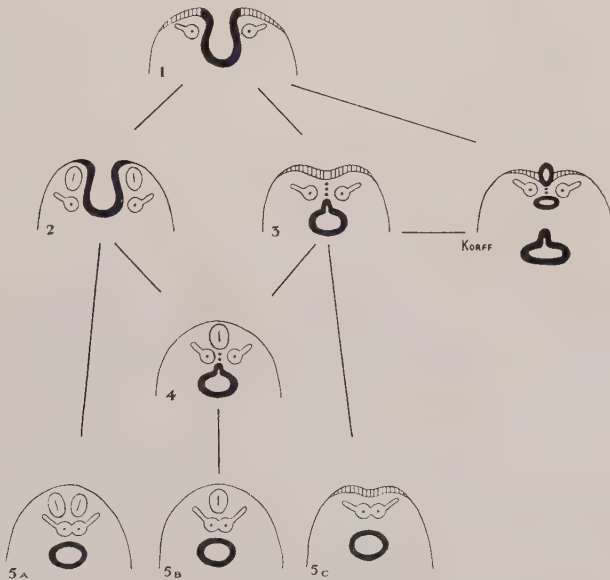


Fig. 7 See text (after Feller and Sternberg).

Group 2. The alimentary tract traverses a vertebral cleft and locally divided spinal cord which surround the intestinal field visible on the back. Representative cases — Adelmann, Lucksch (i), and the present case. (Adelmann's case was a calf with a cleft lumbar column. The large intestine herniated dorsally forming an entodermal field embraced by the divided spinal cord.)

² It is now well nigh impossible to secure the original descriptions of many of these cases; abbreviated accounts may be obtained from the papers of Gruber, Feller and Sternberg. Certain authors have described more than one case; the small Roman numerals indicate the case referred to in those papers.

Group 3. Subgroup (a). The central nervous system remains open as in group 1. A cleft-traversing strand or diverticulum composed of ecto- and entodermal derivatives connects it with the alimentary tract. *Subgroup (b).* Those cases in which no such connection exists. Representative cases — (a) Gruber (i), Risel (i) (iii), Veraguth (i) (?). (b) Lucksch (ii), Gruber (iii), Spanner (i), Svitzer, Gaddi.

Group 4. Subgroup (a). A cleft-traversing strand or diverticulum connects the alimentary tract and spinal cord. *Subgroup (b).* Those cases in which no such connection exists. Representative cases — (a) Risel (ii), Bell, Budde, Feller and Sternberg (ii), Rindfleisch (?), Muscatello (?), (b) Damann, Spanner (ii), Feller and Sternberg (i).

Korff's case, which falls into group 3 and links groups 1 and 3, had an open medullary plate, cleft in two. Within this fissure lay an S-shaped intestinal loop, similar to the ileum in structure, and blind at either end. An ileal diverticulum was directed toward it and the vertebral cleft, but no strand connected the two. It is of interest in that Korff thought it proved Feller and Sternberg's theory. It is discussed later.

Feller and Sternberg's theory embraces certain malformations (fig. 7, 5a, b, c.) more or less closely related to the groups under discussion. These, although at first sight examples of posterior spina bifida, show signs of a former ventral (vertebral body) cleft as indicated by imperfect fusion of the vertebral halves.

Embryology

Previous theories. The genesis of anterior and combined spina bifida has chiefly interested German workers; Gruber ('26) has outlined their earlier views. Hertwig's classical essay "Urmund and Spina Bifida" (1892) had a profound effect on subsequent opinion, since the "ring"³ embryos he produced in the frog were rather like these cases. Concluding

³The alimentary tract, embraced by notochordal halves, opened upon the medullary plate.

that the frog's blastopore closed by a caudally directed fusion of its lateral lips, he regarded a vertebral cleft as due to an incomplete fusion of the lateral blastoporic lips.

Budde thought that Hertwig's work was applicable to man, and Grosser regarded such cases as fall in groups 1 and 2 as proof of this fusion or concrescence theory. Adelmann believed that the defect in his specimen (very like the present case) was essentially the same as these spina bifida or "ring" embryos.

Recent workers such as Korff, Feller and Sternberg, discount the fusion theory as inapplicable to human abnormalities. Budde ('26), changing his views, suggested that a per-

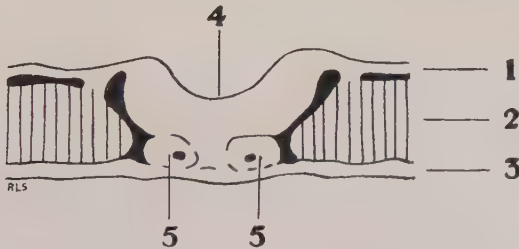


Fig. 8 Diagram based on a micro-photograph by Kolmer.
 1. Ectoderm 4. Medullary Plate
 2. Mesoderm 5 and 5. Notochord
 3. Entoderm

sisting primitive streak would serve as an abnormal entodermal connection and lead to the formation of a dorsal alimentary opening and a vertebral cleft.

Feller and Sternberg suggested that a primitive knot cell rest persisted in the midline causing a notochordal cleft, and that this in turn caused the vertebral centra to be laid down in two independent halves. The subsequent behavior of the cell rest and medullary plate was deemed responsible for the different types met with. In support of this they cited Jablonowski who had observed primitive knot cell rests in the chick, and Kolmer who had reported a double notochord in a 14-somite cat embryo (fig. 8). Korff believed his case confirmed this theory since he thought it could only be explained

by the differentiation of a cell rest which had persisted and formed an organ!

Suggested theory. Feller and Sternberg's theory rests as yet upon somewhat slender evidence. They must however be credited with focussing attention on the cleft condition of the notochord. Notochordal rests were demonstrated in the vertebral halves of both their cases, and Budde found a forked notochord in the cleft region of his case. In the present case nuclei pulposi were found between both the right and left hemivertebrae. In addition, it is of interest that Johnston ('31) has described a partial duplicity of the notochord in an 11-mm. human embryo.

Until further evidence is available it would seem safer to regard a double or cleft notochord as the starting point of these abnormalities. An adhesion of entoderm and ectoderm between the two portions of the notochord (fig. 8) would then account for groups 1 and 2 provided the adhesion underwent penetration. Such an adhesion would also account, according to its degree of persistence, for groups 3 and 4 and their subdivisions. The ultimate form of all would thus depend on the behaviour of the ento-ectodermal adhesion and the medullary plate, for the vertebrae would be laid down in halves. Partial fusion of the halves would explain groups 5*a*, *b*, *c*.

Such an adhesion, linking ectodermal and entodermal cells, would produce either a "neurenteric" strand or traction diverticula at one or both points of attachment. The fistulous openings observed in these cases are possibly due to a foetal necrosis which brings about penetration of the adhesion.

In certain cases of "posterior spina bifida" bony or cartilaginous processes project into the spinal canal, and, dividing it more or less completely into two compartments, are attended by a varying degree of division of the spinal cord. Such cases appear to be related to groups 5*a* and 5*b*; their processes are probably due to partial fusion of the vertebral halves, and appear to be homologous with the "accessory half arches" of the present case.

Application of suggested theory to the present case. The features of the present case may therefore be ascribed to a notochordal cleft and an attendant ento-ectodermal adhesion which interfered with the fusion of the sclerotomes and resulted in a series of hemivertebrae.

It is unlikely that the hemivertebrae were first laid down and then followed by a herniation of the gut, for each spinal cord division had a central canal and was embraced by the ipsolateral hemivertebrae. In other words the adhesion must have undergone penetration with consequent division of the medullary plate and growth of the sclerotomes about those divisions as they underwent closure; because normally the processes of mesodermal segmentation and medullary plate closure march together, and the sclerotomes usually surround the notochord and neural tube simultaneously.

The neural phenomena about the cleft were possibly due to unequal division of the medullary plate, rotation of the left spinal cord division, and displacement of developing nerve roots by the growing "hernial" loop. The medial set of nerves of the left cord division probably account for the presence of the accessory half arches.

The derangement of intestinal rotation may also be ascribed to the formation of the ento-ectodermal adhesion, now represented by the fistula at the apex of the hernial loop. Since a colonic loop was involved the adhesion must have implicated either mid- or hindgut, but suppression of the inferior mesenteric artery suggests that the adhesion was in fact with the hindgut.

Dott ('23) regarded the sequence of intestinal return from umbilical cord to abdomen as the chief determinant of the second and essential stage of rotation. Here the restraining effect of the adhesion must have resulted in unduly early return of the caecum (entering limb), and caused it to take up its position alongside the hindgut (returning limb). The rest of the midgut loop (small intestine), unable to displace the anchored hindgut to the left, disposed itself in front and to the right of the hernial limbs.

The case shows many features characteristic of a nonrotation of the midgut loop, and the position of the small and large intestine approximate to the alimentary tract of the eighth week as portrayed by Dott (fig. 9).

The herniation of the colonic loop is explained by the dorsal intestinal wall growing and pushing its way through

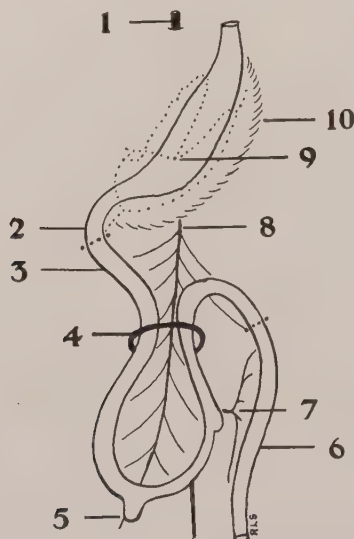


Fig. 9 The alimentary tract in about the eighth week, after the first stage of rotation has been accomplished (after Dott).

- | | |
|------------------------------|-----------------------|
| 1. Aorta | 6. Hindgut |
| 2. Foregut | 7. Inf. Mesenteric A. |
| 3. Midgut | 8. Sup. Mesenteric A. |
| 4. Umbilical Orifice | 9. Coeliac Axis |
| 5. Vitelline Duct and Artery | 10. Great Omentum |

the dorsal gap provided by penetration of the ento-ectodermal adhesion. More precisely, there has been a prolapse of that part of the dorsal intestinal wall forming the cephalic wall of the gap. The muco-cutaneous junctions are indicated in figure 1 by points A and B.

GENERAL FEATURES

Sex and longevity. Like sacral (true) anterior spina bifida most cases of combined spina bifida have been females (fig.

10). Cases of the former or sacral type may attain adult life, but the combined type seldom permits the subject to survive long after birth.

Although the majority of cases of combined spina bifida have been of teratological interest, a number have survived for some time. The life span has varied from a few months to a year or more, and, sometimes, almost a decade, as evidenced by the author's case and those either collected or described by De F. Willard ('04), Bell ('23), Budde ('11), Oehlicker ('09), Greig ('29), Cooperstock and Elzinga ('37).

Oehlicker's case was $6\frac{1}{2}$ years old when x-rayed because of a spinal curvature. It had an "anterior spina bifida" at the cervico-dorsal junction and an attendant posterior defect not unlike that of the present case. Cooperstock and Elzinga's case, a young girl aged 7 years, also had a spinal curvature. A radiograph revealed a defect even more extensive than that of the present case, and an attempt to form a double neural arch. It is not unlikely therefore that their case too had a duplicated spinal cord; such has been found in quite a number of apparently healthy subjects (Bruce, McDonald and Pirie, '05, '06).

Greig's case, an 8-year-old female congenital idiot, had an extensive posterior rachischisis and, because a buttress of bone divided the spinal canal, probably a bifid spinal cord. The anterior defect had undergone reparative changes as evidenced by several malformed and cleft vertebral centra which lay side by side (fig. 7, 5a).

Regional incidence of the defect. Figure 10 is a graphic representation of thirty-seven cases of combined spina bifida. The extent of the vertebral cleft, and the related part of the alimentary tract, is shown for each case.

The chart indicates the number of cleft vertebral bodies, but the extent of the posterior defect could not be plotted owing to the incomplete nature of the records. The cases were arranged in the order shown since they appeared to fall in several groups. Dotted lines indicate that precise information was not available.

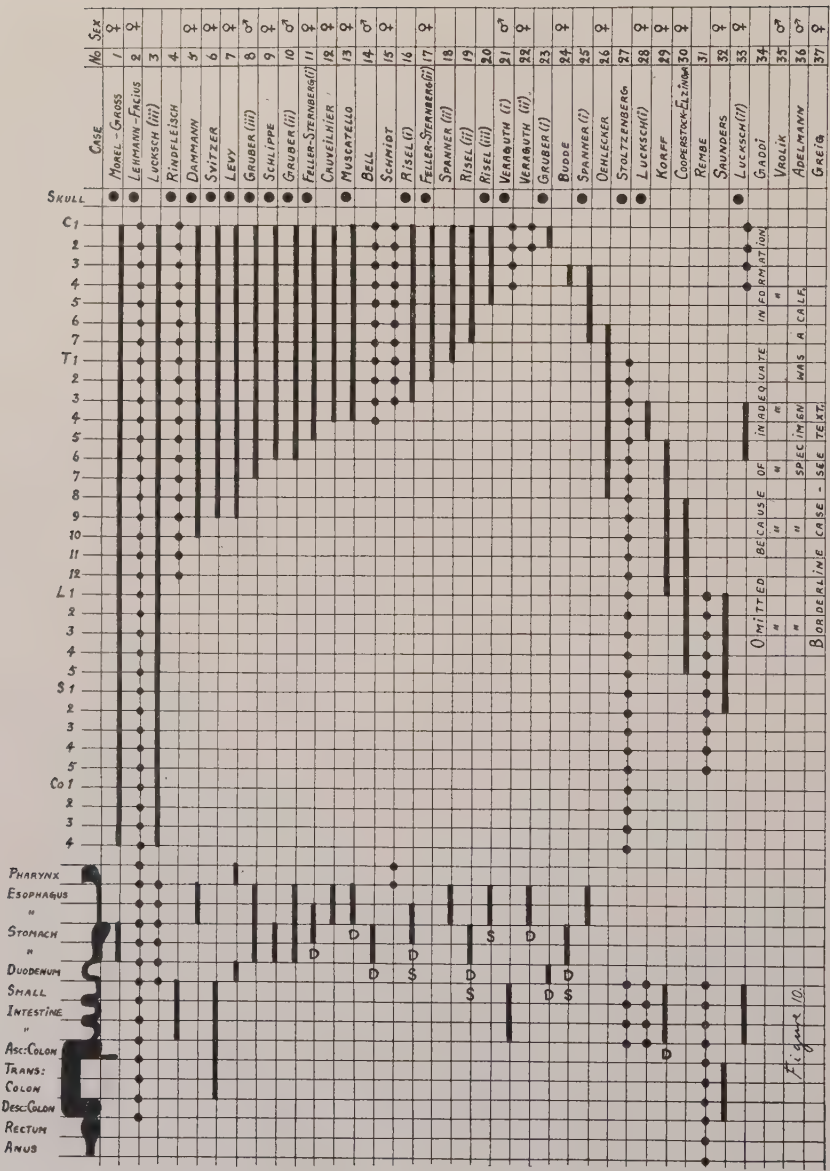


Fig. 10 Graphic summary of recorded cases of combined spina bifida.

It will be noticed that in the majority the cleft either extends throughout the whole length of the vertebral column, or else involves the cervicodorsal and cervical regions in varying degree. In the rest, apart from the cleft commencing lower down, it is interesting that few have been found involving the lumbar region. In contrast posterior spina bifida is per se commonest in the lumbosacral region.

As seen from the chart most of the cases had a cranial defect. This generally took the form of an anencephaly (acrania) or cerebral hernia.

Degree of alimentary involvement. This part of the chart indicates the alimentary region (e. g. gastric, gastro-esophageal) associated with the cleft in each case, and thereby the part of the embryonic gut initially involved. The parts of the alimentary tract most commonly affected are derivatives of the fore- and midgut, which is not surprising considering the cervical and cervicodorsal frequency of the cleft.

In conformity with Feller and Sternberg's classification the related part of the alimentary tract did not in every case open or present itself dorsally. In a few cases it simply lay within the cleft; in others a "neurenteric" connection (e. g. a diverticulum (D), diverticulum and strand (D + S), or strand (S)) passed from it to the cleft (where a few ended) and central nervous system.

The fact that the cleft in most cases commences at the cephalic end of the column and extends caudally for a varying distance, suggests that the defect is not only initiated at an early stage of development, but also overcome with varying rapidity as growth proceeds. As for those cases in which the cleft commences at some distance from the cephalic end of the column, they seem to indicate that the processes bringing about the defect may be initiated at a later date and yet be overcome. From what has been said, it will be appreciated that these statements should be considered in terms of the notochord etc., in that we are dealing with a condition which must necessarily manifest itself in the pre-somite stage of development.

In many respects, all would appear to be modified structural expressions of a common initiator, that, to follow Stockard ('21), merely selected different moments at which to bring about a developmental interruption. As Stockard has pointed out, the sensitive period or "critical moment" of an organ coincides with the high activity which attends its inception. So if we may justifiably draw a close relationship between this condition and a disturbance of the notochord, its greater incidence in the cephalic half of the vertebral column is only to be expected, in that the first formed and consequently most easily affected portions of the notochord would naturally constitute the anlage for that region.

SUMMARY

1. In the lumbar region of a 2-day living human female a mucosal mass projected dorsally, overhanging a small fistula, and was proved by catheterisation and radiography to be large bowel opening through a vertebral cleft. Feces were passed per fistulam et anum. Death occurred in the fifth month.

2. Dissection showed that the colon communicated with the fistula through a cleft extending from the first lumbar to the second sacral vertebra, and through a corresponding spinal cord cleft. Two sets of nerves arose from the left hemicord, one set from the right.

3. Because of vagaries of nomenclature, and to avoid confusion with true anterior spina bifida, cases like this should be called anterior and posterior or combined spina bifida.

4. A survey of comparable cases allocates this specimen to Feller and Sternberg's group 2, which hitherto contained only two specimens.

5. After discussing embryological theories of causation the author proffers a modification of Feller and Sternberg's, suggesting an ento-ectodermal adhesion rather than a primitive knot cell rest, and relates this theory to certain types of "posterior spina bifida."

6. In thirty-seven cases of combined spina bifida the regional incidence, sex, longevity, extent of the vertebral cleft, and degree of alimentary involvement are summarised.

ACKNOWLEDGMENTS

I am indebted to the University of Edinburgh for permission to publish this paper, the substance of which formed part of a thesis accepted for the degree of M. D. My sincere thanks are due to Col. W. F. Harvey of the Royal College of Physicians Laboratory, Edinburgh, for generously placing the material at my disposal; Prof. D. Mainland for his kind advice and helpful criticism; and Dr. R. L. Langley and Mr. H. Stewart, both of Bradford, England, for clinical notes and radiograms.

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MAINTENANCE OF THE PREGNANCY
INDUCED IN THE FEMALE RABBIT FOLLOWING
TREATMENT WITH PITUITARY
GONADOTROPINS^{1, 2}

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The fertility of ova ovulated experimentally by means of gonadotropic materials was demonstrated very early in the studies of pituitary endocrinology. Engle ('27, '31) mated immature female mice that had received implants of fresh pituitary tissue and later found as many as twenty-nine placental sites in a single animal. In no case, however, was an unusually large number of young born. At first he surmised that uterine crowding was the cause of embryonic death, but he later postulated a deficiency of progesterone. Since that time several workers have tried to increase litter size by the use of gonadotropic material, but in general these trials have been unsuccessful.

Cole ('37) and Evans and Simpson ('40) found that a large number of embryos began development in immature rats which had been treated with gonadotropins and then mated. Some very large litters were born, but considerable embryonic mortality occurred and the average litter size was below that of normal mature rats. Cole observed a slightly increased average litter size in adult rats treated with pregnant mare serum during the latter part of the estrual cycle, but he had evidence also of impairment of fertility in some animals.

Casida, Meyer, McShan, and Wisnicky ('43) working with cattle, and Parkes and Hamond ('40), Loginova and Lopyrin

¹ Paper no. 320 from the Department of Genetics.

² Aided in part by a grant from the Wisconsin Alumni Research Foundation.

('38), and Lopyrin, Loginova, and Babicev ('40) with sheep observed multiple pregnancies following gonadotropic hormone treatment, and the latter two groups of authors reported the birth of as many as five normal lambs. Adequate data on embryonic death in these experiments are not available, although the embryos of one of the cows treated by Casida, et al., were resorbed between the fiftieth and seventieth days of gestation.

We have observed (Casida and Warwick, '41) multiple pregnancies in ewes following superovulation induced with pituitary gonadotropins at the time of natural estrus, but embryonic mortality was extremely heavy and most of the embryos died during the first month of pregnancy. Similar results were secured with juvenile female rabbits (Murphree, Warwick, and Casida, '41). The present work is an attempt to identify some of the factors involved in the maintenance of artificially induced pregnancies.

PROCEDURE

Both juvenile and adult-estrual females were treated with gonadotropic extracts of sheep pituitary powder to produce superovulation. They were then artificially inseminated, and the subsequent course of the artificially induced pregnancies studied. Additions of ovarian hormones and other substances suspected of being helpful in maintaining pregnancy were given to some females following insemination and their effects studied. Also, the viability of ova from treated females when transplanted into normal females has been studied.

Each female was treated with a total of 2.5 gm.-equivalents of follicle-stimulating extract³ given subcutaneously in five equal, daily injections and 1 gm.-equivalent of an unfractionated extract³ administered intravenously on the sixth day. Artificial insemination with 0.5 cc. of semen was performed

³ Extracts prepared by Dr. W. H. McShan, Department of Zoology, University of Wisconsin. Follicle-stimulating extract prepared by the method of McShan and Meyer ('40), and the unfractionated extract by a method developed by McShan (unpub.).

within an hour after the intravenous injection. The semen was collected by the use of an artificial vagina, and was usually taken from a sample obtained by pooling the ejaculates of several normal males.

The juvenile females ranged from 80 to 90 days of age and weighed from 1400 to 2200 gm. at the time of the first injections. The adult females were of mixed breeding and of varying ages, but all were sexually mature as shown by a willingness to accept the male when teased at the time of the first injection. They were not mated at this time.

For comparison, experimental pregnancies have been dated from the time of the intravenous injection and normal pregnancies from the time of mating. This probably can introduce no more than a small error since ovulation in the normal rabbit occurs approximately 10 hours after mating and Boyarsky (Wis. Agr. Exp. Sta. unpub.) has recently shown that ovulation is probably complete in less than 15 hours following the intravenous injection in treatments of the type used here.

The treatment with supplementary substances and the transplantation techniques will be described with the results.

RESULTS

Of the average of 25.3 recovered eggs from sixteen juvenile females with no supplementary treatment, 81.2% were cleaving in an apparently normal manner when examined 2 or 3 days following the intravenous injection (table 1). The eggs were considered normal if the blastomeres were of approximately equal size and no fragmentation was apparent. The remaining eggs were either unfertilized, cleaving in abnormal manner, or fragmenting. Although the initial fertility from normal untreated adults has been found to be somewhat higher than this (Murphree, et al., Wis. Agr. Exp. Sta. unpub.), the data indicate that a high percentage of the ova from follicles artificially matured and ovulated in juvenile females are fertilizable and capable of cleaving normally for a time.

An average of 28.2 eggs or embryos was found in seven females autopsied during the period from 5 to 9 days follow-

ing the intravenous injection, but only 37.3% of these embryos were judged to be normal. Many embryos failed to reach a blastocyst stage and were present as fragmenting eggs. Nine females were allowed to go longer than 9 days, but only one of them was pregnant at 10 to 15 days as determined by palpation or exploratory laparotomy. The one female had nine implantation sites at 9 days and produced three young at term.

The data show rather conclusively that under these experimental conditions the juvenile female rabbit can be induced to ovulate large numbers of ova of which a high percentage are fertilizable. The high initial fertility was followed by

TABLE 1
Artificially induced pregnancies in juvenile rabbits.
(No supplementary treatment.)

DAY OF PREGNANCY	NUMBER OF FEMALES EXAMINED	AVERAGE RECOVERY PER FEMALE	
		Normal embryos	Unfertilized eggs or degenerate embryos
2-3	16	20.6 $\pm$ 4.8 ¹	4.7 $\pm$ 2.8
5-9	7	10.5 $\pm$ 1.4	17.7 $\pm$ 6.9
Term	9	0.33 $\pm$ 0.3	

¹ Standard error.

heavy embryonic mortality during the first 9 days of gestation, and virtually no embryos lived longer than 10 days.

The course of pregnancy during the first 12 or 13 days in the treated adult females (table 2) is similar to that shown for the juvenile females. The average of 31.0 fertilized eggs representing 75.4% of those recovered from twenty-six adult estrual females examined the second or third day of gestation shows that, just as in the juvenile female, a high percentage of the eggs are fertilizable. At 9 days only two of six females had normal embryonic sites, and at 12 to 13 days six of ten females showed evidence of embryos having implanted, but only four of these had living embryos. Although the sampling errors are large, this seems to indicate that at least a few embryos will live for a somewhat longer time in the

adult than in the juvenile female, but that by 12 days the greater part of them will have died. None of these females were allowed to go to term so it is not known whether any of them would have produced young. In view of the mortality that was apparently occurring at about the time of autopsy, it appears doubtful if many would have done so.

To determine whether embryonic death was occurring precipitously at any one stage of gestation or whether it was a continuous process all through early pregnancy, a rather careful study of some of the stages between 3 and 9 days was made. The stages selected for most intensive study were the fifth and sixth days because of the ease of recovering blastocysts at those times. The 7-day stage was also included, but did

TABLE 2

*Artificially induced pregnancies in adult estrual rabbits.
(No supplementary treatment.)*

DAY OF PREGNANCY	NUMBER OF FEMALES EXAMINED	AVERAGE RECOVERY PER FEMALE	
		Normal embryos	Unfertilized eggs or degenerate embryos
2-3	26	31.0 ± 5.1	10.1 ± 2.9
9	6	5.0 ± 2.9	2.7 ± 1.7
12-13	10	2.9 ± 1.7	$.9 \pm 0.6$

not prove to be very satisfactory because blastocyst removal was difficult and in many cases the larger ones had to be dissected out. This procedure undoubtedly resulted in the loss of many fragmented eggs and smaller blastocysts. Since there seems to be no data in the literature on the amount of variability in blastocyst characteristics in normal rabbits, a series of normal females was mated and killed at the fifth, sixth, and seventh days of pregnancy to furnish controls for comparison with the experimentals. The diameters of the blastocysts from both treated and normal animals were obtained by placing them in physiological saline in a watch glass under a low-power microscope and measuring with an ocular micrometer. At the time of measurement the blastocysts also were examined for signs of degeneration. Some irregularities suspected of being degenerative changes were observed, but

in almost all cases they probably also could have been induced by injury in removal or examination. The only definite cases of degeneration were in a few very small blastocysts where the size in itself was unmistakable evidence of retardation. Even in relatively small blastocysts, the cell outlines were usually visible with no evidence of necrosis, and they would have passed as normal in younger age groups. The lack of suitable criteria for deciding whether embryos of any particular size class, even if retarded, are still capable of further development or whether they must be considered as inviable makes a fair evaluation of the data (table 3) difficult, and

TABLE 3
Comparison of blastocyst size between normal and treated females.
(Development at 5 to 7 days.)

NORMAL				TREATED			
Female no.	No. frag. eggs	No. blastocysts	Ave. dia. blastocysts	Female no.	No. frag. eggs	No. blastocysts	Ave. dia. blastocysts
			(mm.)				(mm.)
5 Days							
— 394	0	9	1.4	143R	1	4	.3
— 392	0	9	1.4	AA137	15	16	.6
— 393	0	9	1.0	— 233	10	76	.7
— 417	0	12	1.4	— 323	0	16	.9
....		...	..	— 348	0	1	.7
....		...	..	— 388	12	37	1.0
....		...	..	— 380	1	55	1.0
....		...	..	46F1	57	0	..
Ave.	0	9.3	1.3		12	38	.8
6 Days							
— 397	0	11	2.1	AA122	3	27	2.3
— 400	0	12	2.6	45F1	23	44	1.0
....		...	..	A218	0	31	2.6
....		...	..	— 385	1	34	2.7
Ave.	0	11.5	2.4		6.8	34	2.0
7 Days							
— 390	0	4	5.2	Un.M	8	5	4.2
— 391	0	7	5.2	— 324	5	9	2.9
....		...	..	— 341	1	14	4.9
....		...	..	49F4	5	21	2.4
Ave.	0	5.5	5.2		4.8	12.2	3.6

consequently size is the only thing which will be considered in the discussion.

The data from the 5-day group were analyzed by the analysis of variance technique (table 4). There are highly significant differences between rabbits in both the control and the treated groups. These differences are greater in the treated group, but not significantly so. The within-rabbit variation is almost the same for the two groups. The difference between groups is significant ($P < .05 > .01$) when the between-rabbit within-group variance is used as the error term. The proper error term is in some doubt here, and the one used is probably more conservative than necessary.

TABLE 4

Analysis of variance of blastocyst size in normal and treated rabbits at 5-day stage.

	SOURCE	DEGREES OF FREEDOM	SUM OF SQUARES	VARIANCE	F	LINE USED AS ERROR TERM
A	Total	242	30.7	...		..
B	Between groups	1	7.9	7.9	6.69 ¹	C
C	Between rabbits within groups	8	9.5	1.18	19.67 ²	F
D	Normal	3	1.4	.46	6.57 ²	G
					3.52	E
E	Treated	5	8.1	1.62	32.40 ²	H
F	Within rabbits	233	13.3	.06		..
G	Normal	35	2.6	.07	1.40	H
H	Treated	198	10.7	.05		

¹ Probability .05 — .01

² Probability < .01

The small numbers of females included in the 6- and 7-day groups, as well as the poor recovery in the latter group, make statistical analysis unsatisfactory, but in both cases the average blastocyst size appears smaller in the treated animals than in the normals. In the 6-day groups, however, three of four experimentals exceeded the smaller of the two normals and two equalled or exceeded the larger normal. This is probably a sampling variation and not an indication of the treated and control groups being more alike at 6 days than at 5 or 7 days.

The numbers of fragmented ova recovered in the treated animals correspond roughly with the numbers of unfertilized eggs found at 2-3 days (table 2), and probably indicate that death before reaching a blastocyst stage is not an important factor in reduction of the number of embryos. This differs from the observation in juvenile females.

In view of the recognized fact that embryonic mortality is a factor in the reproduction of normal females, reducing the litter size in some cases and completely eliminating litters in others, it seems reasonable to assume that some of the smaller blastocysts among the controls were already going through changes leading to death. If this assumption is made and some general size range arbitrarily chosen, below which embryos will be considered subnormal, the treated animals present the following picture: (1) Almost all females have a high percentage of small blastocysts relative to the size of the majority of blastocysts from normals, and (2) only about half have an appreciable percentage of normal-sized blastocysts. This classification corresponds closely with the observed later performance of comparably treated females (table 2) in which only about half showed evidence of having had any embryos implant, and in these in most cases only a relatively small number had implanted. The great variability from female to female prevents this being made as more than a tentative representation.

The data support the idea that there is no one place at which retardation and death occur, but that the process is a gradual one, more rapid in some females than in others, and that it begins at least as early as the fifth day. As already pointed out, the data from the juvenile females also seemed to bear out the same concept, with the additional suggestion that in them mortality also occurred before the blastocyst stage is reached.

The corpora lutea in the various females were examined macroscopically at the time of autopsy, and in all but one rabbit at stages up to and including 7 days were thought to be normal in appearance, i.e., size, turgidity, and apparent

vascularization. At the 9-, 12-, and 13-day stages, they had a normal appearance in those females having living young, while in those without implantation sites or with only degenerate embryos, they gave almost unmistakable evidence of regression. Uterine sections made of most of the adult and a few of the juvenile females showed normal progestational proliferation in all females up to and including the 7-day stages and in the females at later stages having living embryos. In the females with no living young at 9 days and later, the uteri in no case showed more than a slight proliferation. The histological condition of the luteal tissue corresponded in all cases to the macroscopic appearance of the corpora lutea. Those which appeared grossly to be regressing showed cell vacuolation and degeneration and connective tissue ingrowth.

To see if uterine environment could be improved and thereby the life of the embryos prolonged, females were given supplementary treatments during and following the gonadotropic hormone treatments. In the juvenile females treatment with supplementary substances was started on the second day of gestation and was continued to autopsy or to term or until the females were found to be non-pregnant by laparotomy or palpation. Sixteen females, treated and inseminated in the manner already described, received daily injections of 1 Rb.U. of progesterone⁴ and of these, four were found to be pregnant at 10–15 days (table 5). Three of these were allowed to go to term, but only two produced young—one each. Although these results are not significantly better than the controls at term, there is some evidence that the number of embryos living until implantation was increased. A higher dosage, 3 Rb.U. per day, was tried on three additional females. Two of these were pregnant at 10–15 days, but only one carried young to term, and she produced only two.

Since small amounts of estrogen have been shown by several investigators to aid in maintaining luteal structure and

⁴ Synthetic progesterone prepared by Dr. A. L. Wilds, Department of Chemistry, University of Wisconsin, according to the method of Speilman and Meyer ('39).

function in hypophysectomized and pseudopregnant rabbits (Robson, '37; Allen, '37), a group of females was given daily injections of 5. r.u. of estradiol benzoate ⁵ to see if the structure and function of the corpora lutea could be maintained and embryo-survival increased. Only two of eleven females gave any evidence of having had embryos implant, and in only one of these were there any living embryos at 10-15 days. The one embryo in this female was resorbed before the twentieth day of gestation. In four cases, including the two females with implantation sites, the corpora lutea gave evi-

TABLE 5

Effects of supplementary treatments on course of experimentally induced pregnancies in the juvenile female rabbit.

DAILY TREATMENT	NO. OF FEMALES	FEMALES HAVING PLACENTAL SITES AT 10-15 DAYS			
		No. of females	Ave. no. placental sites	No. females producing young	Ave. no. young born
None	9	1	9	1	3
1 Rb.U progesterone	16	4 ¹	3.5	2 ¹	1
3 Rb.U progesterone	3	2	6.5	1	2
5 R.U. Estradiol benzoate	11	2	3.0	0	..
1 Rb.U progesterone and 5 R.U. Estradiol benzoate	16	10 ²	7.9	3 ²	1.66

¹ Only 3 of the 4 females having placental sites at 10-15 days were allowed to go to term.

² Only 7 of the 10 females having placental sites at 10-15 days were allowed to go to term.

dence, macroscopically, of greater development than in the controls, but in spite of this only one normal appearing placental site was found.

Estrogen and progesterone have been shown to have a synergistic effect, and although it has not been found uniformly possible to maintain pregnancy in ovariectomized rabbits by their use (Allen, '37), it was thought possible that a combination of progesterone and estrogen might provide the necessary stimulus, in addition to the secretions of the

⁵ Progynon-B.

animal's own ovaries, to maintain pregnancy. Fifteen animals were treated with daily injections of 1 Rb.U. progesterone plus 5 r.u. of estradiol benzoate. Ten of these were pregnant at 10-15 days, and of seven allowed to go to term, three produced a total of five young. The corpora lutea of the animals in this group examined at 10-15 days presented a sharp contrast to the degenerate ones found in most animals receiving no supplementary treatment. In most cases they were as large or larger than in normal rabbits, and in only three was there any suggestion of regression. The relatively small number of implantation sites indicated, however, that embryonic mortality had already been high. Histological examination

TABLE 6

Effects of supplementary treatments on course of experimentally induced pregnancies in adult estrual rabbits. (All autopsied at 12-13 days.)

TREATMENT	NO. FEMALES TREATED	FEMALES WITH PLACENTAL SITES		FEMALES WITH EMBRYOS		
		No. of females	Ave. no. sites per female	No. of females	Ave. no. normal embryos	Ave. no. degenerate embryos
None	10	7	16.4	4	7.2	2.2
Vit. C	5	4	16.1	2	13.0	2.5
Chloretone	4	1	10	0	...	..
Lactogen	6	3	18.7	1	19	5
Progesterone and estradiol benzoate	5	4	16.2	4	4.5	1.5

of the uterus was made in only five cases, but in four of these (including females both with and without placental sites) good progestational proliferation was found at 11 days. There is evidence that the early mortality was decreased by this treatment, but the results at term were little if any better than in the females with no supplementary treatment.

In the adult females supplementary treatments of the same progesterone-estradiol benzoate combination used in the juveniles, a lactogenic extract of sheep pituitary glands, ascorbic acid, and chloretone have been used (table 6).

Five females were given injections of progesterone plus estradiol benzoate. All were autopsied at 10 or 12 days and

four were found to be pregnant, but as in the juvenile females treated in this manner, the number of living embryos was very low. The data are too meager for drawing conclusions, but since four of the five treated females had living embryos as compared to four of ten in the controls, there is a suggestion that embryonic life may be prolonged in a higher percentage of females with this treatment. The average number of embryos per female was lower than in those with no supplementary treatment, however. The corpora lutea were large and well developed in all females, and histologically the uteri showed good progestational proliferation.

Ascorbic acid has been reported by Pincus and Berkman ('37) as being present in the uterus of the rabbit in fairly high concentration during the early days of gestation. A progesterone-like effect of ascorbic acid upon the uterus has also been reported by Israel and Meranze ('41). These two reports, together with the finding of Erb and Andrews ('42) that blood ascorbic acid was depressed in some species of animals after gonadotropic hormone treatment, suggested that supplementary treatments with this substance during the early days of gestation might be helpful.⁶

Three females received two daily injections of 4 mg. of ascorbic acid from the day preceding the intravenous injection of unfractionated extract until the third day thereafter, and two daily injections of 10 mg. thereafter until autopsy. Two other females received two daily injections of 10 mg. each from the day treatment with follicle-stimulating extract began until autopsy. Only two of these five animals had living embryos at 11 to 13 days. On the basis of embryo survival and condition of luteal tissue and uteri there is nothing about these females to distinguish them from those with no supplementary treatment. The ascorbic acid was apparently without effect.

The drug, chloretone, is known to raise the ascorbic acid level in the blood of animals receiving it, so at the same time

⁶ The tests with ascorbic acid were made with the cooperation of Mr. H. A. Lardy of the Department of Biochemistry, University of Wisconsin.

the ascorbic acid experiment was being run four other females were fed 200 mg. daily of chloretone from the day injections of follicle-stimulating extract began until autopsy. None of these animals were pregnant at 13 days, although one did have ten degenerate placental sites. Other evidence indicated this treatment had a detrimental rather than a beneficial effect. The corpora lutea had almost completely regressed, the endometrium showed no evidence of progestational proliferation, and the uteri were extremely flaccid and lacking in vascularity. The regression of the endometrium was so great that the uterine sections were much like those of a spayed female. From two animals on this treatment killed at 2 days to get evidence on initial fertilization, there was a suggestion of embryo retardation even at this early stage, although a high percentage of the eggs were fertilized.

Lactogenic extracts of sheep pituitary glands have been reported by Evans, et al. ('41) and other investigators, as having a helpful effect in maintaining the function of experimentally-produced corpora lutea in the rat. To test for this function in the maintenance of pregnancy in the rabbit, six females were given different dosages (two each on daily dosages of 250, 500, and 1000 mg.-equivalents) from the second day following insemination until autopsy. There were living embryos in only one of these, although three had implantation sites. The corpora lutea were not maintained either macroscopically or histologically in three of the other five (questionable in two), and the results were about like those in the untreated females. Whether lactogenic extracts do not act to maintain corpora lutea in the rabbit or whether the dosages were insufficient is not known.

In a further attempt to test the normality of the eggs from artificially matured and ovulated follicles, separate from the environment of the genital tract of the treated female, transplantations of the fertilized ova were made into the uteri of normal females. The hosts were normal estrual females that had been mated to vasectomized males of proven sterility at the time the treated individuals received their intravenous

injection. Three days later the treated animals were killed, the eggs flushed out with physiological saline or rabbit blood serum, and picked up in a small glass pipette. The uterine horns of the hosts were exposed by laparotomy, the pipette forced through the uterine wall, and the eggs discharged with as little of the flushing fluid as possible. As a check upon technique, fertilized eggs from normal untreated females at comparable stages were transplanted into similarly prepared host females. The technique was relatively crude and the rates of success with both types of embryos were low (table 7), but the data suggest that the artificially-ovulated eggs are as viable after transplantation as normal ones. There was evidence of some embryonic resorption late in gestation in both groups.

TABLE 7
Survival of embryos from normal and treated donor-females when transplanted into normal female hosts.

DONOR FEMALE	NO. HOST FEMALES	TOTAL NO. EMBRYOS TRANSPLANTED	APPROX. TOTAL NO. PLACENTAL SITES 10 - 15 DAY: ²	NO. HOST FEMALES BEARING YOUNG	TOTAL NO. YOUNG BORN
Treated juvenile	13	106	20	5 ¹	11
Normal mature	8	42	8	3	3

¹ One other female with one implantation site died at laparotomy.

² Based on laparotomy information in some cases and only on palpation in others.

DISCUSSION

At least two causes can be suggested to account for the high intra-uterine mortality. The first alternative is that in females receiving gonadotropic hormone treatment the uterine environment is inadequate for the young, either as a result of some primary insufficiency on the part of the reproductive system or as a result of some general metabolic disturbance. This could be due to an inability of the female to maintain the experimentally induced corpora lutea in a functional condition. Of other possible deleterious factors, mostly unknown, competition among the large number of embryos might be suggested. The second alternative is that the ova themselves

are abnormal and incapable of developing into viable embryos. The basis for this could be nuclear changes induced by the rapid maturation of the follicles, or the ova could be "unripe" and ovulated with an insufficient store of food material to provide for the early nutrition of the embryos.

The balance of the evidence points toward the first alternative as the major cause of death, although the possibility of the second cannot be definitely excluded. Three lines of evidence point toward this conclusion.

1. Degenerating corpora lutea were observed in many of the females by the ninth to twelfth day of pregnancy. This is sooner than in the normal pseudopregnant rabbit, and may indicate some basic defect or insufficiency in the structure or function of the artificially induced corpora lutea.

2. The treatments with progesterone and with combinations of progesterone and estradiol benzoate apparently prolonged the life of some embryos in the juvenile females and possibly in adult animals. The most logical assumption is that they improved the uterine environment and thereby prolonged the life of the embryos.

3. The artificially matured and ovulated ova survived as well after transplantation into normal host females as did naturally ovulated ova from normal females. In spite of the low rates of success in transplanting both types of ova, a slightly higher percentage of the artificially ovulated ones survived in host females than did in treated females allowed to go to term.

The corpora lutea appeared to be normal in all except one of the adult females autopsied at 7 days and earlier, and their uteri showed a normal progestational proliferation, but in spite of this there was evidence of embryo-retardation before this time. Allen ('37) has shown that the degree of proliferation is not a good index of the capacity of the uterus to support embryos. In view of this, the most probable reason for the death observed by this time is some uterine insufficiency, possibly induced by physiological disturbances outside the reproductive tract and not disclosed by the histological ex-

amination. The possibility remains, however, that defective embryos could account for some or all of the deaths.

Even in the females given supplementary treatments only a small percentage of the embryos survived. This is most readily explained on the basis of chance differences in position or nutritional conditions of the different embryos. However, it can be an indication of inherent differences in vigor.

SUMMARY

Gonadotropic extracts of sheep pituitary powder were used to induce superovulation in juvenile (80 to 90 days of age) and adult-estral female rabbits. All females were artificially inseminated following treatment.

Sixteen juvenile females examined 2 to 3 days after insemination had an average of 20.6 normal embryos, and a group of seven examined at 5 to 9 days had an average of only 10.7. Only one of a group of nine females allowed to go to term was pregnant as late as 10 days, and she produced only three young.

The adult-estral females performed similarly. Twenty-six females examined 2 to 3 days after insemination had an average of 31.0 normal embryos, but this had declined to 5.0 in six females examined at 9 days and 2.9 in ten females examined at 12 to 13 days. No adults were allowed to go to term. A careful examination of 5-, 6-, and 7-day stages indicated that death did not occur precipitously at any one time, but that embryonic retardation was a gradual process, beginning as early as the fifth day in some females.

In attempts to prolong embryonic life supplementary treatments of progesterone, estradiol benzoate, or a combination of the two were given to juvenile females, and a progesterone-estradiol benzoate combination, lactogenic hormone, ascorbic acid, and chloretone were given to adults. The progesterone and progesterone-estradiol benzoate treatments had a beneficial effect as indicated by the fact that sixteen of thirty-five juvenile and four of five adult females receiving them were pregnant 10 to 15 days after insemination. The performance

of twelve juvenile females allowed to go to term, however, was little if any better than that of juvenile females with no supplementary treatment.

To test their viability in a normal uterine environment, 106 fertilized eggs from treated females were transplanted to normal females. Forty-two fertilized eggs from normal females were also transplanted to test the efficiency of the transplantation technique. Only 10.0 and 7.1% of the transplanted eggs from treated and normal females, respectively, survived to term. There is no reason to believe there is any difference in the viability of the two groups of eggs.

The various lines of evidence support the tentative conclusion that embryonic death is due either to a primary or secondary uterine deficiency rather than to defects induced in the ova by the rapid maturation of the follicles.

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DEGENERATION OF THE CORPORA LUTEA IN THE PREGNANT VITAMIN E-DEFICIENT RAT¹

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In the rat, corpora lutea remain functional throughout pregnancy. This is indicated by the fact that bilateral ovariectomy invariably terminates gestation (Astwood and Greep, '38, Evans and Burr, '27, Hain, '34, Harris and Pfiffner, '29 and Johnson and Challans, '30). Failure of gestation similarly occurs in rats maintained on a vitamin E-deficient diet. The corpora lutea of pregnant vitamin E-deficient rats were accordingly compared to those observed in similar stages of pregnancy in animals maintained on a natural food ration or vitamin E-deficient diet supplemented with alpha tocopherol in an attempt to determine whether fetal death in the vitamin E-deficient rat might not be due to failure of luteal function.

Sixty-six female rats of the Long-Evans strain were selected at 4 months of age for the present experiment. These animals consisted of the following three groups: (1) twenty-seven rats which had been maintained on a vitamin E-deficient diet³ from 21 days of age and which had undergone one resorption gestation, (2) twelve litter mate controls similarly

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² Abbott Laboratory Fellow in Biology.

³ Casein, commercial precipitated with HCl..... 27
Cornstarch (cooked) 35
Salts (McCullum 185) 4
Lard 22
Cod liver oil (Squibb) 2
Brewer's yeast 10

The mixed diet without the cod liver oil is allowed to stand for 2 weeks at room temperature to permit the rancid substances of the lard to destroy incipient traces of vitamin E. The cod liver oil is added just before feeding.

treated but given 12 mg. of alpha-tocopherol on the day of positive mating, and (3) twenty-seven animals of similar age and weight but maintained on a natural food ration. Autopsies were performed on the eighth, twelfth, fourteenth, sixteenth, and twentieth days of gestation in addition to autopsy on the day of littering in the natural food or alpha-tocopherol series or on the twenty-third day in the vitamin E-low group. One of each pair of ovaries was fixed in Bouin's solution, and serial sections prepared, stained with hematoxylin and eosin; the alternate ovary was placed in 10% formol and stained for fat with Sudan III. Whole mounts of mammary glands were also prepared.

For the first 16 days of pregnancy no significant difference was observed in the size of the corpora lutea of vitamin E-deficient rats in contrast with those observed in animals maintained on a natural food ration or vitamin E-deficient diet supplemented with alpha-tocopherol. Subsequent to this period the corpora regressed in the vitamin E-deficient animals but continued to enlarge in animals maintained on a natural food ration or vitamin E-deficient diet supplemented with alpha-tocopherol. This enlargement may have been caused to some extent by growth of the extra-luteal constituents of the corpus, but was produced chiefly if not entirely by growth of the individual lutein cell. Histologically, regression of the corpora lutea in the vitamin E-deficient animals was correlated with reduction in size of the lutein cell and a marked increase both in the number and size of lipoid granules within these cells, a finding which Long and Evans ('22) interpret as indicating functional impairment of the lutein cell. Regression was not observed in the natural food or alpha-tocopherol series before the twentieth day of pregnancy. (See table 1.)

Attempts to prevent fetal resorption by replacement therapy with estrone, progesterone, or lactogenic hormone were unsuccessful. Daily doses of 150 International Units of estrone, 4 mg. of progesterone or 2 mg. of lactogenic hormone (50-60 International Units) were injected subcutaneously from the sixth to the sixteenth day of pregnancy. Each group

consisted of four animals. At autopsy on the sixteenth day all fetal sites were resorbing. Histologic examination revealed that the corpora lutea of animals receiving estrone or progesterone were as degenerate as those observed in untreated controls. The corpora of lactogenic treated animals were well developed and exhibited no evidence of regression or degeneration; all fetal sites nevertheless were resorbing.

It is significant that degeneration of the corpora lutea in the pregnant vitamin E-deficient rat invariably occurred subsequent to the death of the fetus and the fetal placenta.

TABLE 1

Size of the corpora lutea at different stages of pregnancy in the rat.

DIETARY GROUP	NUMBER OF ANIMALS	DAY OF PREGNANCY	MEAN DIAMETER OF CORPORA LUTEA IN MM.
E-low	3	8	1.31
E-low	3	12	1.54
E-low	3	14	1.58
E-low	6	16	1.76
E-low	6	20	1.63
E-low	6	23	1.47
Normal	3	8	1.28
Normal	3	12	1.47
Normal	3	14	1.63
Normal	6	16	1.76
Normal	6	20	2.05
Normal	6	20-23	1.70
Alpha-tocopherol	4	16	1.74
Alpha-tocopherol	4	20	1.98
Alpha-tocopherol	4	22-23	1.73

In the rat, as has already been pointed out, corpora lutea remain functional throughout pregnancy. This is indicated by the fact that bilateral ovariectomy invariably terminates gestation. However, pregnancy may continue to term in animals hypophysectomized after the eleventh day. (Pencharz and Long, '33). This indicates that some extra-pituitary factor is responsible for the luteal function of late pregnancy. For

the first half of pregnancy maintenance of luteal secretion is controlled by the anterior pituitary, and more specifically, by lactogenic hormone (Cutuly, '41, and Lyons, Simpson and Evans, '43); during the second half, luteal control is taken over by an extra-pituitary factor, elaborated, according to Astwood and Greep by the fetal placenta. It is precisely this structure which remains abortive in the vitamin E-deficient rat (Selye, Collip and Thomson, '33).

Inasmuch as death of the fetuses and the fetal placenta invariably preceded degenerative changes in the corpora lutea, and inasmuch as administration of progesterone and lactogenic hormone (at least in the dosage used) failed to prevent fetal death, it is believed that the degenerative changes of the corpora lutea in the vitamin E-deficient rat were the result of rather than the cause of fetal death.

In agreement with the findings of Urner ('30) mammary glands grew normally in the vitamin E-deficient rat to the eighteenth day of pregnancy. Subsequent to this period they regressed in the vitamin E-deficient rat but continued to enlarge and lactate in animals maintained on a natural food ration or a vitamin E-deficient diet supplemented with alpha-tocopherol. Mammary involution was observed in the pregnant vitamin E-deficient rat only after the death of the fetal placenta.

SUMMARY

For the first 16 days of pregnancy no significant difference was observed in the size of the corpora lutea of vitamin E-deficient rats in contrast with those observed in animals maintained on a natural food ration or vitamin E-deficient diet supplemented with alpha-tocopherol. Subsequent to this period the corpora regressed in the vitamin E-deficient rats in contrast with their continued growth in animals maintained on a natural food ration or vitamin E-deficient diet supplemented with alpha-tocopherol. Histologically degenerative changes were evident in the corpora lutea of vitamin E-deficient rats as early as the sixteenth day of pregnancy. Regres-

sion of the corpora lutea in the vitamin E-deficient rat invariably occurred subsequent to the death of the fetal placenta.

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DISTRIBUTION OF DRY CONSTITUENTS OF YOLK AND ALBUMEN IN THE DEVELOPING AVIAN EGG

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ONE FIGURE

The proportional amounts of dry matter in yolk and albumen of the developing avian egg are not constant but undergo various changes. These changes are brought about by general metabolism and functional activities of accessory embryonic organs or membranes — allantois and amnion. The dry matter of yolk and albumen is, therefore, closely associated with the water movements within the egg, especially during the formation and disappearance of the embryonic membranes.

In recent years considerable amount of data has accumulated concerning both the water-metabolism and the concentration of solids in the developing chicken egg (Needham, '31; Romanoff and Romanoff, '33). In this brief report observations have been extended to include two other species, Ring-necked pheasant (*Phasianus torquatus*) and Bobwhite quail (*Colinus virginianus*). The dry matter content of the dense yolk and of albumen of these species, and the data on liquefied yolk of chicken egg (*Gallus domesticus*) are included in this study. All quantitative determinations of dry matter were made by methods previously described (Romanoff, '30).

EXPERIMENTAL RESULTS

Dense yolk

As may be observed from figure 1 the changes in dry matter of the dense yolk of the developing egg of all three species

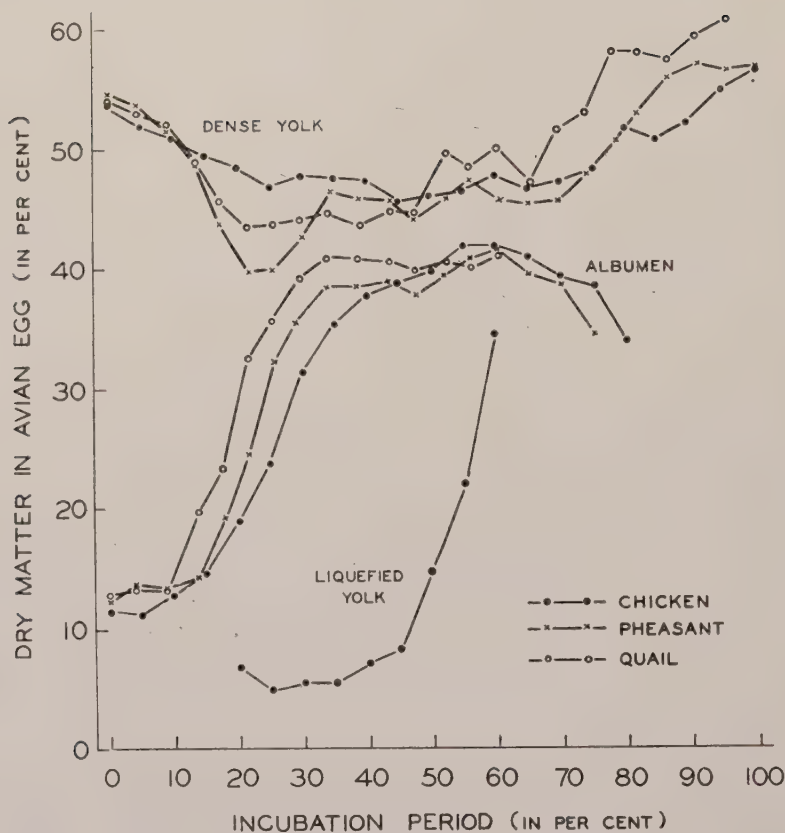


Fig. 1 Changes in dry matter of dense yolk and of albumen of the developing eggs of White Leghorn chicken, Ring-necked pheasant and Bobwhite quail, and of liquefied yolk of the chicken egg /plotted against the incubation age expressed in percentage of total period — 20 days for chicken and 23 days for pheasant and quail/.

studied followed in general a definite pattern. From the original value of about 54%, the dry matter decreases to a low point of 45%, and remains at that point until the latter part of incubation. Then it rises slowly to a value slightly higher than that of the original, or to about 57%.

Liquefied yolk

With the initial appearance of liquefied yolk in the egg (Romanoff, '43) its dry matter content is very low, averaging

not more than 5%. Then with the decrease in volume, the proportional amount of dry matter begins to rise sharply. In the chicken egg, for example, on the thirteenth day of incubation the liquefied yolk is already no longer present as a chemical entity (Shklyer, '37), and its dry matter content is identical with that of dense yolk.

Albumen

The dense layer, that is, the most predominant component of the albumen, shows a marked rise in dry matter content during the first third of the developmental period, from about 12 to 40%. Then it nearly levels off with only a slight decrease, to about 35%, at the time of its entire disappearance, or union of its remnants with the yolk sac. The curves of these changes (fig. 1) are essentially identical for the eggs of all three species.

DISCUSSION

The content of dry matter in yolk and albumen is reciprocal to the water content of these egg constituents (Romanoff, '30). This reciprocity or close relationship is such that in the dense yolk the moisture increases as the dry matter decreases, while the total weight remains practically constant throughout most of the incubation period (Romanoff and Romanoff, '33). With the loss of water from the albumen there is an increase in dry matter content, but in this case the total weight of albumen first rapidly, then gradually diminishes.

SUMMARY

The dry constituents of yolk, dense and liquefied, and of albumen in eggs of chicken, pheasant and quail undergo changes during incubation according to a definite pattern, which presumably is identical for all avian eggs.

The percentage of dry matter in dense yolk falls at the beginning, and then rises at the end of incubation, that of liquefied yolk is almost unchangeable except a sharp rise at the time of disappearance from the egg, and that of albumen rises sharply and after long inactivity has some fall at the time of merging with the yolk.

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BOOK REVIEW

THE ANATOMY OF THE FEMALE PELVIS by C. F. V. SMOUT, with sections on The Histology of the Female Reproductive Tract, and a chapter on Ovarian Endocrine Function by F. Jacoby. Foreword by Sir Beckwith Whitehouse. Edward Arnold and Company, London, July 1943, 0/35/0 net.

The assembling of the fundamentals of the anatomy and physiology of the female pelvis into a well illustrated compact volume will be welcomed by all medical students. In so doing Professor Smout has rendered a service to the pedagogy of Gynecology and Obstetrics. That it was done under the stress of war will make this volume all the more appreciated. One can not but praise the wisdom of the British Government, in these days of priorities, for permitting its publication, in color.

Professor Smout, assisted by his able collaborator Doctor Jacoby, has produced a concise and amply illustrated book with 170 figures, in the compact limit of 190 pages. Artist Pardoe shows promise in anatomical illustration. His figures are simple but show depth, a valuable aid in fixing a forgotten subject in the mind at a glance. The book is intended primarily as a refresher for the medical student just entering upon the study of Gynecology and Obstetrics. But the midwife should find this volume more than profitable reading, she might even consider it essential.

Each chapter, on the viscera, includes a brief but adequately illustrated section on development. Chapters I, II, and III, on Bones and Joints, have 42 illustrations in 29 pages. The text points out the significance of the anatomy as it occurs. The treatment of chapter I, Bones of the Pelvis, is admirable, but the space given to a comparison of male and female pelves were better utilized elsewhere, in so brief a treatise; for the male pelvis is of little moment here, and could be handled in a short paragraph.

Chapters IV and V, The Walls of the Pelvis and Pelvic Fascia, seem to be the least ably handled in the book, especially the fascia. It is to be regretted that such a finely planned volume should be deficient in so important a phase of pelvic anatomy. Perhaps the author thought a more adequate discussion was beyond the scope of the book. The reviewer would suggest that the space above indicated

as superfluous, in the chapter on Bones, be used in amplifying the chapter on fascia. In these same chapters occur figures which will mislead the student. Numbers 43 and 51 give unreal ideas of the pelvic floor, while figure 52 confirms the text in confusing *Arcus Tendineus Fasciae Pelvis* with *Arcus Tendineus M. Levatoris Ani* (BNA).

The Ovary and its Histology comprise chapter VI. Twelve of the 15 sections illustrated are of excellent human material. They span the years from prepubertal to premenopausal time and include both gravid and non-gravid sections. The discussion is adequate. It is fitting, at this time, that endocrine functions should be reviewed so thoroughly, for the gynecologist needs to be hormone conscious, and the time allotted to this subject in many physiological laboratories is minimal. To this might be added a short appreciation of the importance of psychical degeneration following bilateral oophorectomy in the absence of adequate substitution therapy, especially in younger women.

An excellent Chapter VIII, on the Uterine Tube and on Ovulation, follows. Here, a short discussion of tubal and other ectopic conditions would enhance the reader's perspective. Chapter IX, The Uterus and Vagina, is one of the best in the book. Figure 120, a cervico-vaginal section, and the text which applies to it, may be studied with profit by all beginning gynecologists. It shows the overlapping zone between columnar and squamous epithelia. The implications are discussed. The next chapter treats of the important relations of the Genitourinary and Alimentary Tracts. This is followed by a chapter on the External Genitalia. Both are adequately figured and discussed.

Chapter XII, The Perineum is especially good. Illustrations 144, 145, 146, 147, and 148 form a series which might well serve as a pedagogical model for the review of any anatomical region. Figure 149, veins of the sacral vertebral canal, is timely in view of the recent advances in caudal anesthesia.

Chapter XIII, the Pelvic Lymphatic System, is well treated descriptively. It would be rendered more useful if illustrations of special regions were added. A short discussion of the physiology of the lymphatics as made out by Webb and his colleagues would not be amiss, for the factors brought out by them may yet prove to be significant in the dissemination of carcinoma.

Chapter XIV, The Innervation of the Pelvic Viscera should be the most enlightening chapter of all for the preclinical student. It is a discussion of the gross physiology of the pelvic organs. In an age in which stress is laid on microchemical researches from specialized laboratories, to the exclusion of much gross physiology, the student often approaches his clinical studies with little knowledge of many

aspects of physiology. This chapter is an excellent pioneering venture in teaching. A less diagrammatic rendering of figure 151, the innervation of the pelvic viscera, would have improved this section.

Chapter XV, considers the usual facts of Placental Formation. Among several figures, numbers 160, 161, fetal and maternal aspects of the placenta are excellent. But figure 153, a schema of placenta circulation, leaves much to be desired. Figures 162, 163, 164, and 165, types of placental vessels, and 166, a section of the umbilical cord deserve careful study by any student. The last chapter, XVI, on Fetal Circulation, has a convincing photograph of the great vessels of a full term fetus. This figure emphasizes the spatial relations of the ductus arteriosus and its significance in postnatal changes. In this connection mention might well be made of the gross anatomical studies of Noback and Rehman. Great discrepancies in the literature exist concerning this structure, and its physiology is of significance to the obstetrician as well as to the internist.

A more comprehensive index will increase the usefulness of this volume. One might wish that in such a recent publication the BNA might have been adhered to, at least in parenthesis, for colloquial terminology limits in a small way the handiness of this very useful book. At least this is true for the Canadian and the American medical student. Certain figures, 43, 48, 51, 57, 88, and 151, might be rendered with greater accuracy of detail, since they cover areas important enough to warrant less diagrammatic treatment. In addition the following changes are suggested for future editions: more sections both coronal and transverse; better treatment of the suspensory ligament of the ovary and its contents; additional and improved figures of the arterial and venous blood supply of the uterus, tube, ovary and vagina; and a reminder to the student of the necessity of checking the cervix at menopause for carcinoma.

In conclusion it is hoped that differences of emphasis by the reviewer, are in no way to be interpreted as detracting from the value of this excellent compact work. It stands as a helpful volume for all about to enter upon a study of the anatomy and gross physiology of the pelvis. In pioneering this difficult field of anatomical pedagogy, Professor Smout, his collaborator Doctor Jacoby, and their illustrator, Mr. Pardoe, deserve well earned praise. We need other concise illustrated small volumes of specific anatomical regions, with the gross physiological implications emphasized.

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PLEURO-PERITONEAL MEMBRANE AND BURSA INFRACARDIACA

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THREE TEXT FIGURES AND ONE PLATE (FIVE FIGURES)

The pleuro-peritoneal membrane is familiar to embryologists as one of the elements participating in the formation of the mammalian diaphragm; the infracardiac bursa, named and described by Broman ('04), is a small, flattened, mesodermal cavity located in the right pleural ligament, inconstantly present in the adult. The relationship between these two structures seems remote, yet it will be shown that they are the only remaining members of a considerable series, the others of which are recognizable in early embryos but soon lost. The membrane is formed by the growth and fusion of the rims or edges over a shallow niche or depression which is thus converted into a flattened diverticulum of the coelomic cavity, closed above, open below. The bursa is the upper tip of such a diverticulum which has secondarily been cut off from the parent cavity. Both are derived from niches or diverticula in the wall of the coelom and it is in this form that others of the series appear.

The term niche seems to me preferable for these structures, since diverticulum connotes to my mind a tubular outgrowth, actively growing out from a fixed point in the wall of a cavity (one exception would be Meckel's diverticulum and there are doubtless others) whereas in the present case the tubes increase in length by the progressive closure of the walls over a depression, the tip remaining fixed while the mouth

or opening moves caudally. At least niche describes the usual basic shape.

In the human embryo, on which these studies are chiefly based, the coelom in the form of pericardial cavity extends forward in early stages almost to the level of the oral plate, the aortic trunk issuing from it beneath the first and second pharyngeal pouches. It connects with the peritoneal cavity by two dorso-lateral passages, called variously the pericardio-peritoneal, pleuro-pericardial or pleural passages or canals, and by Streeter ('42) simply the coelomic tracts. The tracts thus lie ventral to the region of the pharyngeal pouches and are coextensive with the latter as they successively develop. The niches appear in the walls of the tracts and are at first in direct relation with the pouches.

In mammals the number of paired pharyngeal pouches is characteristically four. The first pair arise from the pharynx by broad bases and point dorso-laterally, the second point directly laterally, the third and fourth are directed more ventro-laterally and produce, moreover, branches (the future thymus elements) pointing medially. These latter seem to attract the coelomic mesothelium which responds by sending out toward them hollow, horn-shaped diverticula with thickened epithelium, later broadened out to become shallow niches. The first two pouches produce no corresponding niches, but I have numbered the niches for their respective pouches, beginning with number 3.

The whole series of niches is shown in the drawings (figs. 1 and 2). The larger drawing, based on reconstructions, represents the lateral body wall of the right side, above the umbilical cord, of a human embryo of 4 mm., seen from within; the heart, liver and median fore-gut are removed. The broad diagonal septum transversum showing a cut surface separates the pericardial and peritoneal cavities, which are joined above its free border by the coelomic tract. The aortic conus issues from the pericardial cavity, and above it appear the lower edges of the four pharyngeal pouches. Under pouch III and along the tract the niches are found; another is shown in re-

lation with the lung bud on the lateral wall of the fore-gut (fig. 2). It will be seen that in a 4-mm. embryo five pairs of niches may be recognized. I have followed the custom commonly in use in regard to the aortic arches of skipping the questionable fifth and numbering the pulmonary arch the sixth; niche 6 therefore follows niche 4 in the series. Two

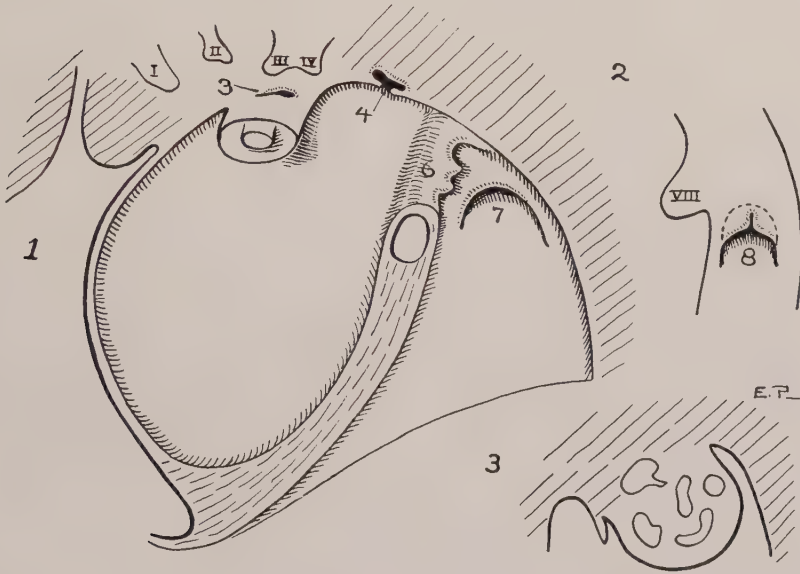


Fig. 1 Median view of the lateral wall, above the umbilical cord, of a human embryo of 4 mm., showing the position of the series of niches in relation to the septum transversum and the coelomic tract. Full description in text. Diagram compiled from reconstruction.

Fig. 2 Portion of fore-gut of same embryo to show niche 8 in relation with lung bud, VIII.

Fig. 3 Dorsal pillar of Uskow shown as pointed ridge on mesial wall of Wolfian body. Diagrammatic drawing from transverse section of embryo of 10 mm.

niches have developed without corresponding pouches, showing that, in spite of the intimate relations in the early stages, the pouches are not a necessary feature. The full series of niches seems to substantiate the theory of the loss of pharyngeal pouches in mammals and to mark the lung buds as the

caudalmost pair of pouches, following in their position on the ventral pharyngeal wall the successively more ventral trend of the others.

Niches 3 and 4 are first seen in an embryo of 2.4 mm. (figs. 4 and 5). All photographs are from human embryos in the Harvard Embryological Collection. This embryo was sectioned in utero and the plane is oblique, so that the section reproduced needs explanation. The spinal cord is seen above and the fore-brain below, the latter continued into a small slice of the side of the mid-brain. The right side of the embryo is broken and shows the open mouth cavity ventral to the head and the open coelom with torn walls. Below (i.e. ventral to) the spinal cord lie the notochord with dorsal aortae on either side and the median pharynx, connected on the left side with the large fourth pouch. Oblique sections of the lateral sides of the third and second pharyngeal pouches can be seen in series toward the angle of the mouth, in relation with the gill clefts on the outside of the head. The small circular epithelial structure median to pouch II is the thyroid diverticulum, in relation with which the irregular, endothelial tube of the ventral aorta can be recognized. Its mesothelial coat, assumed as it enters the pericardial cavity to become conus, bulges between grooves, marked by the two lower arrows. The upper two arrows point to the diverticula from the coelomic tract which are to become niches 3 and 4.

These two structures are not merely folds of the coelomic wall, for they are present in only a few sections of the series. They interdigitate closely with and lie caudal to their respective pharyngeal pouches. As is shown in higher magnification (fig. 5) their walls are of thickened epithelium, and the more caudal diverticulum shows a crescentic intraepithelial slit, perhaps foreshadowing the cavity of the niche. In one embryo of 4 mm. (fig. 6) niche 4 is in the form of a tiny, open, horn-shaped object, the cavity appearing in only a single section, curving toward the caudal tip of the thymus element of pouch IV, also shown in the section. In another embryo of 4 mm., from which the reconstruction (fig. 1) was made, niche 3 is a

deep dimple with thickened epithelium on the side of the aortic conus, while niche 4 has moved caudally along the pericardial roof and is a shallow rounded cup with overhanging edges. As such it can be recognized in all embryos up to 8 or 10 mm., but after this it fades out completely.

Niche 6 lies on the lateral wall of the coelomic tract directly in line with the duct of Cuvier as it courses from the cardinal veins to join, in the body wall, the umbilical vein and thus gain entrance to the sinus venosus. After the establishment of the umbilical drainage through the liver and the degeneration of the portion of the umbilical vein in the body wall, the duct of Cuvier is freed at this end and is pulled across the upper end of the pleural canal, carrying with it the inner layers of the lateral wall, which then form the pleuro-pericardial membrane. The niche is carried on the crest and pleural slope of the advancing membrane. It takes the form of a shallow pouch, the higher medial rim of which arises from the tip of the membrane so that in section it projects caudally from the crest (fig. 7). The rim is ragged, with several blunt, short fimbriae, and in other embryos of this age has been mistaken for simple exuberant growth, yet by comparison with other niches of the series its true character becomes evident. As the pleuro-pericardial membrane meets the mediastinum to close the upper end of the pleural passage, the niche becomes more and more restricted; the last seen of the rim is a small tab of tissue partially blocking the tiny channel which marks the last point of closure. There it is constantly recognizable in embryos of 16 mm. or older.

Niche 7 becomes covered by the pleuro-peritoneal membrane. As first seen it is a shallow depression at the dorso-lateral corner of the coelomic tract at the level of the lung buds in embryos of 4 mm. Its rim soon begins to overhang (fig. 8), except caudally, and grows over the depression to form the membrane. Caudally the membrane is continued as the dorsal and ventral pillars of Uskow. The dorsal pillar forms a longitudinal ridge on the dorsal body wall just mesial to the posterior cardinal vein and later to the wolffian body

(fig. 3). The suprarenal gland develops mesial to the ridge. The ventral pillar moves from the lateral wall to the dorsal (or cranial) edge of the septum transversum, and thence is carried onto the lateral wall of the dorsal lobe of the liver, which grows from the septum and invades the pleural passage, almost filling it. The top of this lobe is shown in figure 7, dorsal to the rim of niche 6, but not connected with it. The membrane grows longer by the progressive fusion of the edges of the two pillars, and forms, in man, a small lateral part of the central tendon of the diaphragm (Bremer). In some animals it plays a greater part in the closure of this structure. The niche cavity becomes absorbed in the peritoneal cavity.

The history of niche 8 has already been written by Broman ('04) in his description of the infracardiac bursa. Each niche starts as a shallow depression, already present in the embryo of 2.4 mm., on the lateral wall of the outer coat of the foregut, just below the bulging lung bud. Each becomes covered by a membrane formed by the fusion of the overhanging rims, open caudally (fig. 7). The left niche degenerates early and fades out; the right one elongates downward by the continued fusion of the rims until it forms a long, flattened diverticulum, crescentic in section to fit the side of the oesophagus. Its membrane is added to the right pleural ligament, and the diverticulum opens below the diaphragm into the lesser peritoneal cavity. Normally the upper part becomes cut off by the pressure of the diaphragmatic muscles and either completely disappears by absorption or persists as the infracardiac bursa. The lower portion becomes the superior recess of the lesser peritoneal cavity. It is chiefly interesting as the site of right-sided diaphragmatic hernia of the oesophageal-hiatus type, possible when the bursa is not separated from the recess.

In mammals the membrane covering niche 7 is utilized to a greater or less extent in the completion of the diaphragm. In the chick, and presumably in birds in general, the so-called diaphragm is formed by the covering membrane of niche 8, and other niches also differ from the mammalian pattern.

Niche 6 is only occasionally recognizable as a shallow depression with thickened epithelium at the angle between the lateral body wall, and the ridge covering the duct of Cuvier; this position is often occupied merely by two or three short vertical folds of the mesothelium. The two types may be seen on the two sides of the same embryo. Niche 7 can be recognized in chicks of the third and fourth days as a small, cup-shaped, thick-walled depression in the dorso-lateral angle of the coelom at the level of the lung buds. It is not present in all embryos and soon flattens out and disappears. Niche 8 develops during the third day and persists on both sides. Its covering membrane fuses below with the dorsal liver lobe and is carried by it to the lateral body wall, where again fusion takes place. Thus the membrane assumes a frontal, instead of its original sagittal, plane. The lung grows down along the former lateral, now dorsal, surface of this covering membrane, and then fuses by all its surfaces with the coelomic wall, leaving no true pleural cavity. In spite of this fact the membrane, since it lies between the lung and the niche cavity, which opens below into the peritoneal cavity, has been called the "pleuro-peritoneal membrane" (Lillie, '08), a rather unfortunate name since this structure has no relation with the membrane so designated in mammals, which rises from niche 7. The thin, freely movable sheet of tissue often described as the avian diaphragm is actually the surface, or ventral wall of the posterior thoracic air-sac, which soon after hatching spreads from the root of the lung into the "pleuro-peritoneal membrane" and even into the body wall beyond. The sac can be inflated by pressure on the abdominal air-sac or on the liver over it; the sparse musculature of the sheet is derived from the body wall muscles. It is obviously not a true diaphragm.

Five pairs of niches can be recognized in human embryos. Niche 4 apparently best illustrates their original relations to the pharyngeal pouches. As the lateral tip of the pouch meets the ingrowth of the surface ectoderm of the gill cleft, so the ventral branch of the pouch, the thymus element, meets the

outgrowing diverticulum of the coelom. The lung buds possess no thymic branch but are themselves ventral and thus in relation with the coelom, and for two of the niches corresponding pharyngeal pouches do not exist in man, yet the series of niches remains. Mostly they are short-lived and functionless, only the pleuro-peritoneal membrane has been turned to a useful purpose. Their constant presence in embryos, however, confirms the theory of lost pharyngeal pouches in man and helps to establish the lungs as derivatives of the last pair of pouches.

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PLATE 1

EXPLANATION OF FIGURES

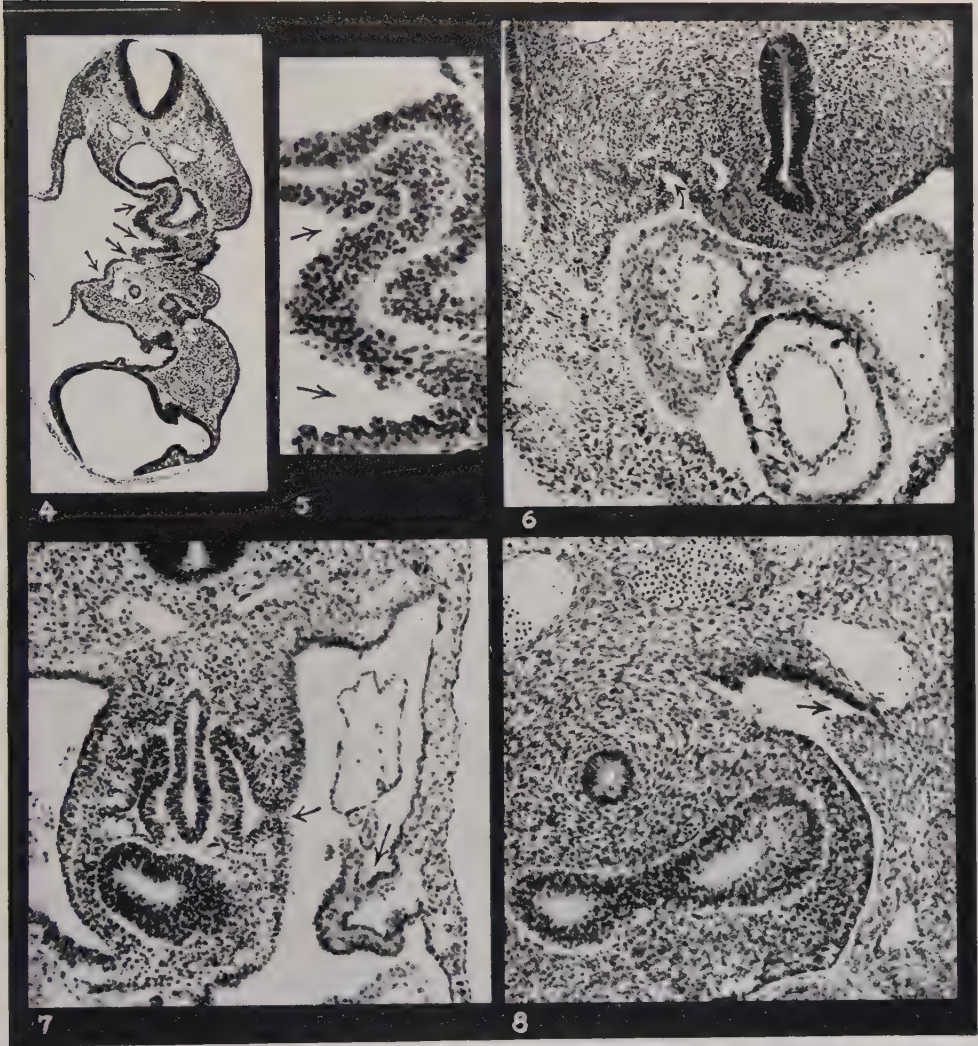
4 Oblique section through head of human embryo of 2.4 mm. Two upper arrows point to niches 4 and 3. Full description in text. $\times 30$ diam.

5 Same section, photograph at higher magnification of niches 4 and 3. $\times 125$ diam.

6 Embryo of 4 mm., transverse section through top of atria. Midline is marked by aortic bulb and flattened pharynx. Niche 4 is the horn-shaped diverticulum from the pericardial cavity (arrow) pointing toward a solid epithelial structure, the lower tip of pharyngeal pouch IV. $\times 70$ diam.

7 Embryo of 4 mm., transverse section through caudal wall of lung bud. Arrows point to niche 6 on the dorsal wall of the partially formed pleuro-pericardial membrane enclosing the duct of Cuvier, and to niche 8 of the left side, behind and below the lung bud. The right member of the pair is already closed over by its membrane. Dorsal to the rim of niche 6 lie its fimbria and the dorsal lobe of the liver. $\times 70$ diam.

8 Embryo of 6.3 mm., transverse section through bifurcation of lung bud. Arrow points to niche 7, in vicinity of posterior cardinal vein on the left side of the embryo; dorsal and ventral pillars are closing over it. $\times 70$ diam.



STUDIES ON THE THYROID GLAND

I. THE STRUCTURE, EXTENT, AND DRAINAGE OF THE "LYMPH-SAC" OF THE THYROID GLAND (*FELIS DOMESTICA*)

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FOUR TEXT FIGURES AND TWO PLATES (EIGHT FIGURES)

INTRODUCTION

Numerous investigations have established that the thyroid glands of most forms possess well developed plexuses of lymphatic capillaries in their capsules as well as within the substance of the glands proper. These channels usually present no marked peculiarities when compared with lymphatics elsewhere; that is, they consist of plexuses of vessels conforming to the contour of groups of thyroid follicles (colloid vesicles) but retaining the proportions of lymphatic capillaries at all times.

In the cat (*Felis domestica*) the lymphatic vessels within the thyroid gland seem seldom, if ever, to be of capillary proportions but exist as a huge, capacious, labyrinthine space, permeating the substance of the gland completely, dividing the parenchyma of the gland into small areas, and separating the capsule from the parenchyma everywhere except at a few sites, usually at points of entrance or exit of vessels and nerves (Ramsay, '38; Ramsay and Bennett, '42 and Ramsay, '43). Earlier investigators overlooked this outstanding structural feature, in the cat, although the thyroid lymphatic vessels of that form have been described.

Frey (1863), Wölfler (1880), and Baber (1880) studied the thyroid lymphatics of the cat, among other mammals, but

limited their descriptions to other forms, intimating the condition in the cat to be not unlike that of the other animals investigated. Navalichin (1874) described large lymphatic spaces, lined with endothelium, in the thyroid gland but did not state the form he studied. Bartels ('01), using Gerota's (1896) fluid, studied the thyroid lymphatics of many mammals, and, although he noted the ease with which the cat's thyroid could be injected, apparently did not appreciate the true proportions and extent, both subcapsular and intraglandular, of the lymphatics. Lewandowsky ('02) noted large lymph-spaces in the thyroid of the cat. Regaud and Petitjean ('05) interpreted the thyroid lymphatics of the cat to be the same as those of the dog except that they were easier to inject. Although Major ('09) included the cat in his comparative study, he, also, failed to recognize its unique structure. Matsunaga ('09) overlooked the correct lymphatic pattern of the cat (included in a study with human, dog, pig, and rabbit) and postulated lymphatic communications between the thyroid lymphatic capillaries and the contents of the follicles. Further, he extended his theory to include intracellular connections, as well.

More recently, Rienhoff ('31) has given an excellent account of thyroid lymphatics in man and dog. Although two cats were also included in his study, the description was based obviously on the dog alone for the conditions present in the cat were not mentioned and were therefore probably not observed. Rossi ('33) included the cat in an extended comparative study of thyroid lymph drainage but was concerned mainly with the gross aspect of the drainage pathways.

The observations listed above (excepting those of Rienhoff), have been rather superficial, usually dealing, in a gross way, with the lymphatic drainage pathways of the gland. No description of the enormous degree of development of the intraglandular ramifications as well as the coextensive subcapsular portion of the lymph-sac, as shown by the cat, has been found by the writers. Likewise, the relatively great number of thyroid lymphatic vessels that drain directly into veins and

into lymphatic trunks without first traversing lymph-nodes, in the cat, has also escaped notice. This condition presents an opportunity for studying in detail the structural characteristics of the thyroid lymph-sac in the cat and, further, suggests many problems dealing with the functional aspects of the gland. The present study is concerned primarily with a description of the structure, extent, and drainage of the thyroid lymph-sac.

MATERIALS AND METHODS

The gross aspects of the extent and drainage of the lymph-sac were studied by carefully elevating the capsule of the gland and injecting, subcapsularly, India ink which had been diluted with 4 parts of distilled water. A standard Tuberculin syringe fitted with a 27-gauge needle was used. These injections were made on cats under sodium amytal anesthesia. In each case the skin of the ventral side of the neck was opened by a mid-line incision that started at a point located 1 cm. caudal to the symphysis of the mandible, and extended to the cephalic border of the sternum. The sternothyroid and sternohyoid muscles were retracted to their respective sides, thus exposing the trachea, larynx, and thyroid gland medially, and the common carotid arteries, jugular veins, vagal and sympathetic trunks, and the cervical lymphatic trunks laterally.

Observations on the drainage pathways were made during the actual injection and immediately thereafter, utilizing careful dissection to follow the lymphatics carrying the injected fluid from the gland. Motion pictures in color were taken of several of the injection procedures.

The finer ramifications of the lymph-spaces within the substance of the thyroid glands were best studied in organs which had been partially injected with a very small amount of normal saline solution, then fixed, embedded in paraffin, sectioned serially, and stained with hematoxylin and eosin and Mallory's connective tissue stains.

Attempts to demonstrate the intraglandular ramifications of the cat's thyroid lymphatics by injection, followed by

clearing the gland, did not produce satisfactory results. The lymph-spaces within the gland were found to be so extensive that, following clearing, the glands were totally opaque with injection medium. If one attempted to remove a smaller piece of these glands for study by transmitted light the injected material drained away, for once the gland was opened the continuous lymphatic space was opened, releasing the injection material.

Both Trypan blue (1%) and India ink (1 part ink to 20 parts of water) were introduced into the lymph-sac of several animals (under ether anesthesia) to test the phagocytic capacity of the lining elements. These animals were sacrificed at daily intervals, from the second day up to 1 week. Another group of cats received repeated subcutaneous injections of Trypan blue, and a final group had several intraperitoneal injections of the same material. The thyroid glands from these animals were fixed, embedded in paraffin, sectioned serially, and mounted with no additional staining.

Silver nitrate solution was introduced into the thyroid lymph-sac of several animals, immediately following death, to outline the endothelial cells lining the lymphatic spaces. Large pieces of the capsules of these glands were mounted in balsam on slides, and portions of thyroid parenchyma (sheets of follicles) were mounted in a similar manner for study (figs. 9 and 10).

OBSERVATIONS

a. The lymph-sac and its drainage. The ink, injected subcapsularly into one thyroid lobe spreads at once through the entire lymph-sac of that lobe, then across to the other lobe by a bold channel marking the region of the original isthmus of the gland. Thus the lymphatic spaces of the entire gland (lateral lobes and the variable isthmus) may be quickly and easily injected by introducing the fluid into either the right or left lobe. (Bartels, '01, noted the ease with which the isthmus in the cat could be injected from either lobe while Rienhoff, '31, using excessive injection pressure, succeeded in injecting both lobes and the isthmus, in the dog, by introducing fluid into

either lobe.) As soon as the needle point enters the cavity of the lymph-space there is an instantaneous spreading of the ink solution, due apparently to capillary attraction within the spaces, even before any pressure is exerted on the plunger of the needle. This phenomenon was especially obvious when a syringe with a loosely fitting plunger was used for injection.

The ink solution, immediately following its introduction into the gland, appeared in the lymphatic vessels that drain the extensive lymph-sac. About .1 to .15 cc. of the fluid was allowed to pass freely into the sac (in large adults), using a very slight pressure on the syringe plunger (barely enough to move it). In such a manner sufficient injection fluid was provided so that the drainage would last long enough for all the efferent vessels to be detected and sketched. Pressure injection and distention of the lymph-sac was never practiced.

There are usually two main groups of lymphatic vessels draining the thyroid gland in the cat. One group, the superior (cranial) group, arises at the superior pole of each lateral lobe and drains to a large, deep cervical lymph-node located dorso-lateral to each lobe (figs. 1, 2, 3, and 4). From two to nine separate vessels, on each side, have been seen to compose the superior group. The second, or inferior, group of vessels arises at the caudal pole of each lateral lobe and drains caudally and caudolaterally into small lymph-nodes located ventral to, and along the sides of, the trachea (tracheal lymph-nodes), to small nodes located in the superior mediastinum (mediastinal nodes), and to nodes of variable size located close to the jugular-subclavian venous junction (jugular lymph-nodes). Compare figures 1 to 4.

In eighteen of the thirty-two animals injected with ink solution it was possible to see thyroid lymphatic vessels pass directly to veins without first traversing lymph-nodes. Such vessels emptied either at the junction of the internal and external jugular veins or at the junction of the subclavian and jugular veins. Also, in addition to the thyroid lymphatics that empty into veins directly, there are, in many cases, lymph-vessels from the gland that pass laterally or caudolaterally

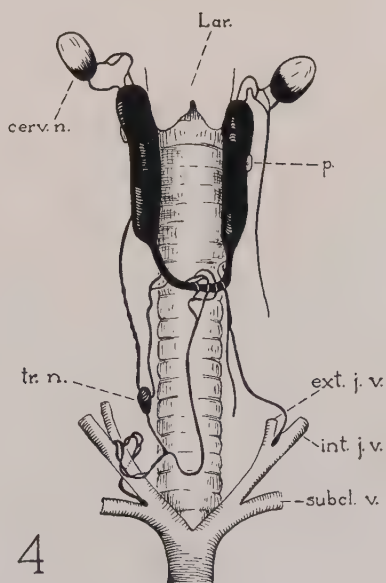
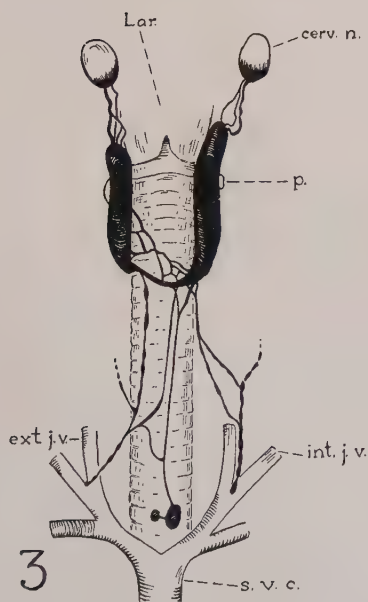
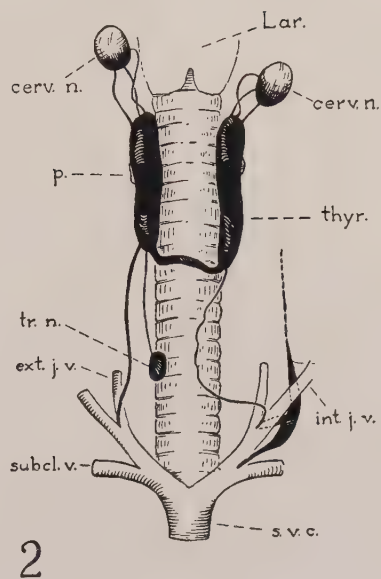
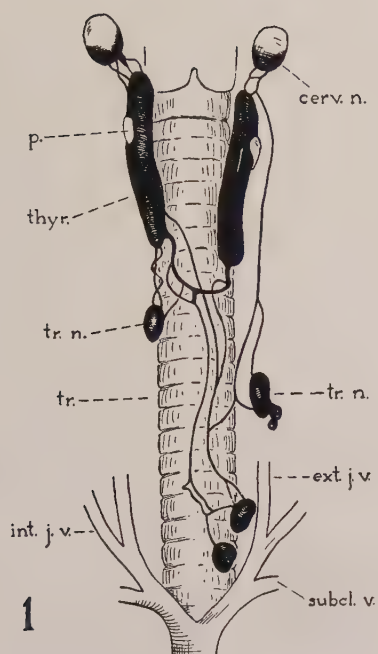
to empty directly into the cervical lymphatic trunks on either the right or left side and, in two animals, into both trunks. These "direct" vessels, whether they go to veins or to the cervical lymphatic trunks, are never the only vessels draining the thyroid but are supported by others which conform to the usual plan (that of traversing lymph-nodes before entering the venous system). The "direct" vessels, described above, usually arise at the caudal pole of the lateral lobes. In only one instance a "direct" lymph-vessel was seen to arise from a superior pole of the gland. Without doubt, if excessive injection pressure had been employed, many more "direct" vessels would have been seen.

There is usually one main channel, connecting the two lateral lobes, marking the site of the original thyroid isthmus. Although a parenchymatous isthmus is usually difficult to demonstrate in the cat, the large lymph-channel, marking its fetal location, persists in the adult as a lymphatic connection between the two lateral lobes. Also, smaller additional isthmic vessels are usually seen (figs. 1 and 3). Many of the caudal group arise from the main channel of the isthmus and, at times, some may arise from the lesser isthmic vessels or from the medial border of the lateral lobes, as well (figs. 1, 3, and 4). Anastomoses between the lymphatic vessels was commonly encountered.

The extent of the intraglandular ramifications of the thyroid lymph-sac is well shown in figures 5, 6, 7, 8, and 12. In the preparation of these specimens a small amount of normal saline solution was injected subcapsularly, thus serving to

Figures 1 to 4. Drawings of injected thyroid glands and their lymphatic drainage vessels from four cats (litter mates, two-thirds grown). The larynx, trachea, and the great veins of the neck are included for orientation purposes. Lymphatic vessels are shown in heavy lines.

cerv. n., cervical lymph-node	p., parathyroid gland
ext. j. v., external jugular vein	subcl. v., subclavian vein
int. j. v., internal jugular vein	s. v. c., superior vena cava
i. v., innominate vein	thyr., thyroid gland
lar., larynx	tr., trachea
	tr. n., tracheal lymph-node



Figures 1 to 4

accentuate slightly the lymph-spaces and render them more easily visible. The capsule adheres to the substance of the gland in very few places. In the specimen illustrated in figure 5, only one connection between the capsule and parenchyma proper can be seen (at the upper right). The broad contact between the thyroid vesicles and the lymph spaces, usually encountered in the cat, is demonstrated clearly in figures 6, 7, and 8. That the intraglandular ramification of the thyroid lymph-sac or lymph-space shown here is not of capillary proportions is obvious.

b. Character of the cellular elements lining the lymph-sac. Silver nitrate preparations show the entire sac (subcapsular and intraglandular portions, alike) to be lined with apparently typical endothelial cells (figs. 9 and 10). It will be seen that the inner surface of the capsule and the outer surfaces of single follicles, as well as bands or sheets of follicles are covered by endothelial cells. Larger blood-vessels (fig. 10), nerves, and the few connective tissue trabeculae that traverse the lymphatic spaces from the capsule to the parenchyma of the gland (figs. 5 and 12), are all covered with endothelium, as would be expected. The entire sac is, therefore, lined with endothelium.

The phagocytic capacity of the lining endothelial elements, tested by dilute carbon ink and Trypan blue, was found to be very slight, if present at all. Only prolonged retention (4 days to 1 week) of the colloidal materials in the sac itself, seemed to stimulate a few of the endothelial cells to store small amounts of those materials. Appreciable dye storage was never encountered. Whether an actual engulfing or just an adsorptive process had occurred was usually difficult to determine because the cells are so thin in vertical section. Also, oblique sections of the endothelial surfaces, upon which there had been adsorption of dye particles (India ink), are extremely difficult to interpret. Thus it may be that the small amounts of carbon particles seen in the slides represented actually an adsorptive rather than a phagocytic process. Free phagocytic cells containing ingested dye particles were encountered in

the lumen only in the prolonged experiments. No dye storage, of any type, was seen in the lining elements (or the free cells occasionally encountered in the lumen of the sac) in those animals which had had either intraperitoneal or subcutaneous administration of the particulate materials. A few intravenous injections of carbon ink and Trypan blue produced similar negative results.

DISCUSSION

In this study an attempt was made to employ only those procedures which would disturb the normal conditions as little as possible. Therefore, inert carbon ink was used for injection and, since one is dealing here with large spaces (not capillaries) a very thin, dilute injection medium was not necessary. Further, because the spaces to be injected are capacious no injection pressure was needed. Obviously, excessive injection pressure would lead to rupture of lymphatic channels and distortion of the true vascular relations. Finally, since the animals were all alive (under anesthesia) during the injection and drainage, massaging the gland was never resorted to for drainage occurred normally. Such crude procedures as excessive injection pressure and squeezing or massaging the gland with resultant damage to small vessels have, in the past, given rise to confusion between veins and lymphatic vessels.

The results of this study, as far as the general gross aspect of the drainage pattern of the thyroid lymphatics is concerned, are in general agreement with the findings on the cat reported by Bartels ('01), Matsunaga ('09), and Rossi ('33) with exception that these investigators failed to see (or hesitated to record) direct connections between the lymphatics and veins (or lymphatic trunks) without intervening lymph-nodes. Direct connections between lymphatic vessels and veins have been proved definitely and, further, direct connections between thyroid lymphatics and the veins of the neck have been described by several investigators. Major ('09), Caylor, Schlottbauer and Pemberton ('27) and Rienhoff ('31), among others,

have described such connections in the dog. Similar direct channels, in the human, have been reported by Mahorner, Caylor, Schlotthauer and Pemberton ('27); Polonskaja ('34) and others.

The criteria for identifying a channel as a lymphatic vessel, in the present study, were several; namely, the origin, termination, histological structure, closely-set valves, and so forth. At first, all such vessels were examined routinely in histological section. It soon became apparent, however, that the most reliable of the criteria were the origins and terminations of the channels in question. Those vessels which never carry blood and which pass directly to the cervical lymphatic trunks, for instance, must be lymphatics and not veins. Also, many vessels, after leaving the thyroid lymphatic sac, branch and anastomose with similar channels, and some of these branches pass into lymph-nodes while others proceed directly to cervical lymphatic trunks or to veins (figs. 3 and 4). Such entire complexes of channels must, obviously, be lymphatic and not venous. Motion pictures in color were taken of many of the injections as an additional method of recording the injections and subsequent drainage patterns for future study and interpretation. These films, when projected, were run backward as well as forward so that one could compare, at will, the drainage vessels during and after injection with the original vascular pattern (before injection). Thus there need be no doubt as to the identity of the vessels in question. It is difficult to understand the failure of earlier investigators to observe the relatively great number of direct lymphaticovenous connections in the cat.

The subcapsular and intraglandular portions of the lymph-sac of the cat are most significant. Here instead of a plexus of lymphatic capillaries there exists a single, continuous, capacious lymphatic space (figs. 5, 6, 7, 8, and 12), obviously not capillary-like or even sinusoidal. It is clear, from the illustrations, that follicular epithelium possesses more contact with lymphatic than with blood-capillary endothelium and that the lymphatic endothelium is separated from the follicular epi-

thelium by a minimum of intervening connective tissue (figs. 6, 7, and 8).

Thyroid lymph, in some forms, has been shown to contain detectable amounts of thyroid secretion. Carlson and Woelfel ('10); Carlson, Hektoen and Schulhof ('25) and Hicks ('26) have demonstrated thyroglobulin in lymph collected from the cervical lymphatic trunks below the entrance of the thyroid lymphatics, in dogs. It would seem certain that the extensive lymphatic spaces of the cat's thyroid gland would play a still more important role in the passage of the secretion from the gland. Early investigators reported the presence of colloid in thyroid lymph and therefore postulated for the thyroid lymph a major role in conducting the secretion from the gland. Occasionally, in this study, colloid has been seen in the lymphatic spaces. In each such case, however, careful study of serial sections disclosed a break in one or more thyroid vesicles with resultant loss of their colloid to the lymphatic spaces. Extreme care must be exercised in handling the cat's thyroid if such breaks are to be avoided. In all probability, any thyroid secretion in the lymphatic spaces would never appear as colloid masses and, further, would be extremely difficult to demonstrate in ordinary histological preparations.

Considerable confusion and misinterpretation of thyroid structure has resulted from the work of Williamson and Pearse ('23 and '30) and Williamson ('26). Among other concepts they have postulated lymphatic spaces, surrounding follicles, having but a "parietal" layer of endothelium, none covering the follicles themselves. As evidence in favor of their hypothesis they presented a photomicrograph of thyroid development in the human fetus (Williamson and Pearse, '30, fig. 336, p. 537). Upon close examination this picture is seen to illustrate the development of a cervical lymphatic node — not thyroid gland — and the lymphatic space shown is the peripheral sinus of the node. They interpret as thyroid lymphatic spaces the cystic epithelial remnants of embryonic material of pharyngeal pouch IV origin. Cervical cystic tracts (occurring from the level of the thyroid gland to that of the thymus

body) as well as cystic epithelial portions of the original thymic anlage, within the thymus body, were interpreted by Williamson and Pearse as lymphatic channels of a "closed thyro-thymic lymph system." The ideas of Williamson and Pearse have never been confirmed (Chouke, Whitehead and Parker, '32). The lymph-sac, in the cat, is entirely different from the lymphatic apparatus hypothesized by Williamson and Pearse and should be neither confused nor compared with it.

The parenchymatous portion of the thyroid of young cats seems to have but few connections with the capsule. Many more areas of connection are seen in adult cats and consequently a complete injection of the gland is a little more difficult than in young animals. It may be that, as age progresses, secondary fusions with resultant adhesions between capsule and parenchyma provide the larger number of trabeculae seen in the gland of the adult animal.

The significance of the thyroid lymphatic space is not clear. Why the cat, among the mammals investigated, is unique in this respect is also confusing. One is reminded of the condition in certain fishes (Ferguson, '11 and Burne, '26) where the branchial lymph, returning to the veins and to the heart, flows through and about the thyroid gland, bathing its follicles with lymph. Developing thyroid parenchyma in the cat (Ramsay, '38) apparently influences the early appearance and great growth of lymphatic endothelium for the typical gland as well as for small ectopic masses of thyroid parenchyma widely separated from the main gland (fig. 11). It would seem probable that such an enormous lymphatic space, together with its intimate and extensive relationship with the follicles, would constitute a pathway, of importance, for thyroid secretion.

SUMMARY AND CONCLUSIONS

1. Methods are outlined for demonstrating the gross and microscopic aspects of the thyroid lymph-sac in the cat.

2. Complete injection of the whole gland could be produced by injection into the lymph-sac of either lobe, whether a parenchymatous isthmus was demonstrable or not.

3. The thyroid lymph-sac is drained mainly by lymphatic channels which pass to deep lymph-nodes of the neck (deep cervical, pretracheal, and jugular).

4. Over half of the animals injected showed thyroid lymph-vessels which passed directly to veins without traversing intervening lymph-nodes. In several cases thyroid lymph passed directly to the cervical lymphatic trunks, without intervening lymph-nodes.

5. The ramifications of the lymph-sac permeate the parenchyma quite completely, providing broad contact between lymph-channel and the follicles.

6. The lymph-sac is lined by typical lymphatic endothelium.

7. The possible role of the lymph-sac and its drainage vessels in transporting a considerable proportion of the thyroid secretion is suggested.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

5 Transection of the right lateral lobe of the thyroid gland of an adult cat. The lymph-space was accentuated by injecting a small amount of normal saline solution before fixation. The capsule is adherent to the parenchyma of the gland only at the upper right. Parathyroid IV containing a small cyst is seen in the interior of the lobe. $\times 225$.

6 and 7 Photomicrographs illustrating the degree of the intraglandular ramifications of the lymph-sac and the relationship of the lymphatic endothelial cells to follicular epithelium. Note the arteriole, covered with endothelium in the right lower center of figure 7. Both figures are taken from the same gland as that shown in figure 5. $\times 225$.

8 The amount of perifollicular connective tissue is illustrated by Mallory's connective tissue stain. Note the arteriole in the center of the field. $\times 175$.

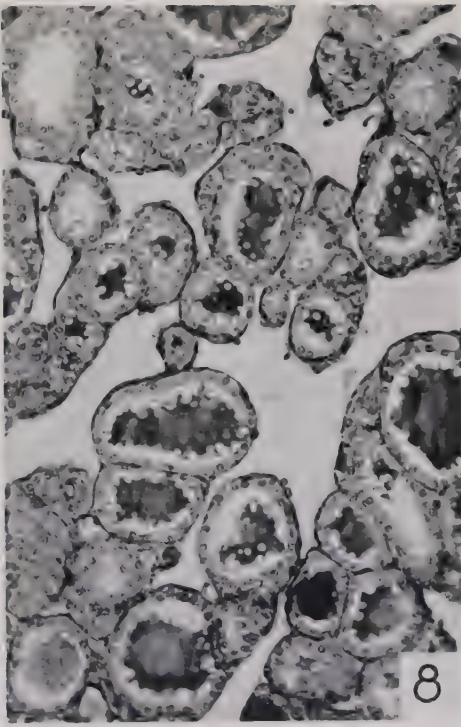
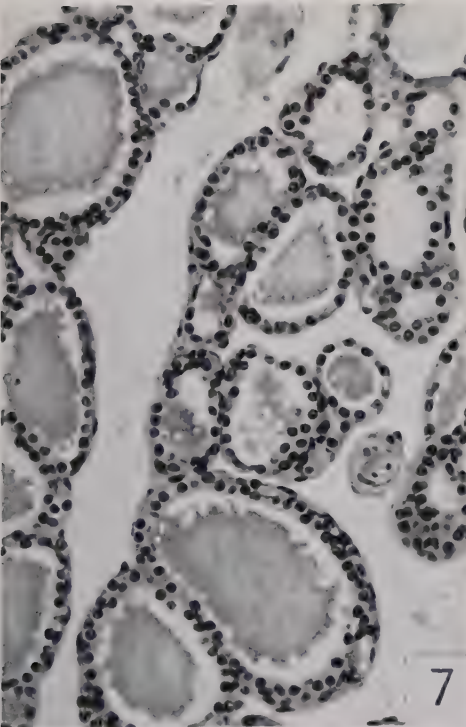
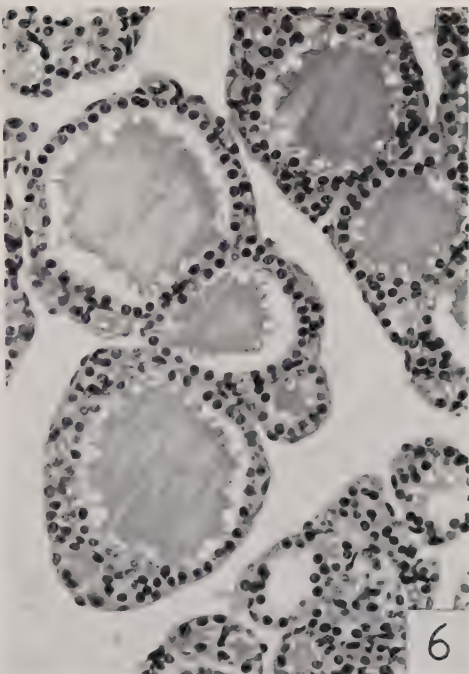
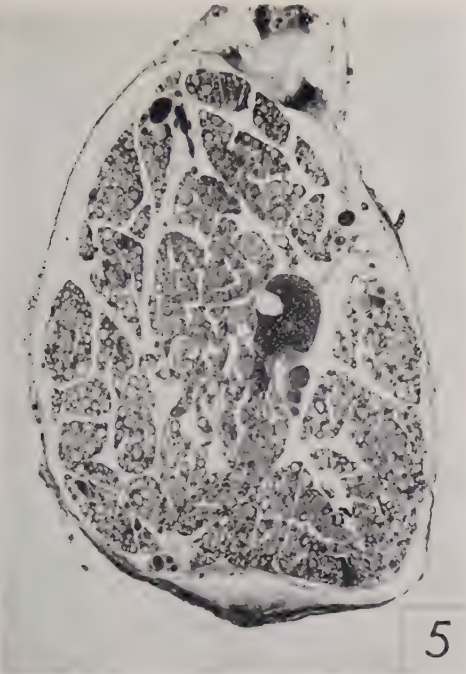


PLATE 2

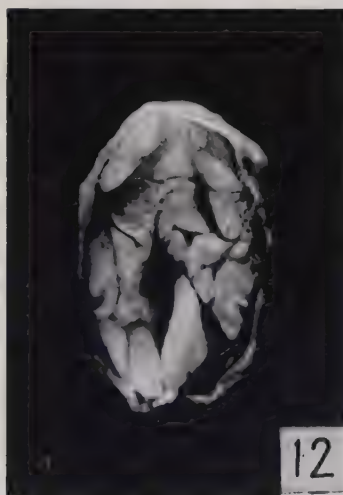
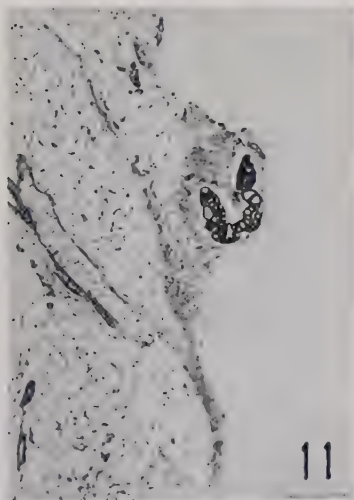
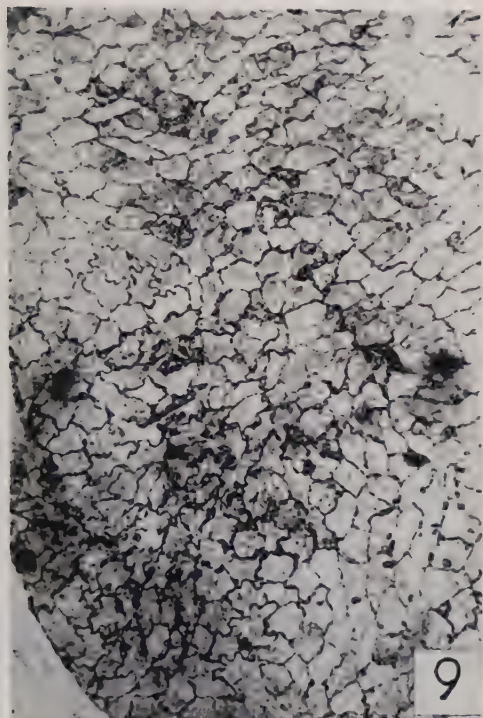
EXPLANATION OF FIGURES

9 Photomicrograph of the inner surface of the capsule of the thyroid gland from an adult cat following treatment with silver nitrate. Lymphatic endothelial cells are clearly outlined. $\times 110$.

10 Surface view of a small piece of thyroid parenchyma from an adult cat. Silver nitrate has outlined the endothelial cells covering a small flat band of follicles (at left) and, also, covering blood-vessels (at right) traversing the lymphatic space. Note the differences in shape and size between the endothelial cells which line the capsule (fig. 9) and those covering the parenchyma and vessels (fig. 10). $\times 110$.

11 Small ectopic thyroid mass taken from the right side of the trachea $2\frac{1}{2}$ cm. below the right thyroid lobe. Note the lymph-space about the follicles. $\times 35$.

12 Photograph of a left thyroid lobe from an adult cat. A moderate amount of normal saline solution was injected into the lymphatic space before fixation (10% formalin). The lobe was transected with a razor blade; no stain was used. Note the broad, continuous lymph-space. A cyst of Complex IV origin is seen in the upper left center of the lobe. Compare with figure 5. $\times 10$.



AN INJECTION METHOD TO DEMONSTRATE THE BLOOD SUPPLY OF NERVES

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ONE FIGURE

Although many papers have been published on the blood supply of ganglia and central and peripheral nerves, it is surprising that the current text books of anatomy either ignore or relegate this phase of morphology to one of negligible importance. The vasa nervorum have long been reported to be present on all nerves. The past work in this field is presented in the excellent review by Adams ('42). Recently attention has again been given to their structure and possible function in neurological studies by Bergmann and Alexander ('41), Bergmann ('42 and '43), Adams ('42) and Bentley and Schlapp ('43a and '43b).

Since the vasa nervorum are small and may not be seen easily in many instances, they may be frequently overlooked. However by using small-particle injection material, such as pigmented, liquid-latex or vinylite, these vessels can be studied with little difficulty. This method permits a study of their origin and entire course and, in this respect, is superior to previous injection or staining methods.

Studies were made on the peripheral nerves of two cadavers, in which the arterial system was injected with red liquid-latex through the femoral artery, and four human head and neck preparations. One of the latter was injected with latex and the other three with vinylite. Red material was injected into the internal carotid artery and blue material, into the

jugular vein of each side. The specimens were subsequently fixed by injecting embalming solution into the soft tissues and body cavities.

Liquid-latex, after being hardened in the vessels by formalin, alcohol, or acid fixing solution, still maintains its elasticity and permits easy dissection of the injected vessels. Vinylite, on the other hand, forms a hard cast of the vessel when hydrated and the finer branches of vessels filled with this substance may be broken, if not dissected carefully. A syringe with a glass cylinder and rubber piston should be used for injecting these substances, since latex or vinylite may cause the piston of "all glass" or "all metal" syringes to adhere to the cylinder wall.

Although blood vessels were observed on most of the peripheral nerves, particularly in the extremities of the injected cadavers, the study reported here is limited to the demonstration of the arterial supply of the peripheral divisions of the facial nerve, as an example of this injection method.

The arteriae nervorum to the peripheral divisions of the facial nerve arose from arteries nearest to their course in the face and therefore varied with the arterial pattern of the face. However as shown in figure 1, in most of the specimens each nerve division received a fairly large branch from the artery nearest its exit from the parotid gland (superficial temporal, transverse facial or anterior auricular artery) and another branch, near its termination in the facial musculature, from the arteries to these muscles (external maxillary, submental, superior and inferior labial, angular, frontal, parietal or posterior auricular artery). In addition to these main arterial sources, smaller arteries from the general arterial network of the subcutaneous tissue, also supplied each division of the nerve along its course. The arteriae nervorum parallel the course of the nerve for a short distance, pierce and run in the epineurium and subsequently in the perineurium. These smaller arteries then divide into proximal and distal branches which end near to, or anastomose with, adjacent arteries along

the course of the nerve (fig. 1a). The veins though not studied in detail, followed much the same course as the arteries.

Although no attempt is made to relate the arteriae nervorum to facial palsy resulting from exposure, trauma or diseases of the vascular system, since the reports of Adams ('42) and Bentley and Schlapp ('43a and '43b) have indicated that the vasa nervorum influence the conduction of peripheral nerves,



Fig. 1 Dissection of a face to show the size, course and relations of the arteries to the facial nerve after injection of the vascular system with pigmented vinylite. a. Enlarged segment of nerve trunks and arteries.

they should be considered as functional, anatomical structures and studied in relation to the anatomy and physiology of these nerves.

SUMMARY

The blood supply of peripheral nerves can be demonstrated by injecting a pigmented, small-particle substance (liquid-latex or vinylite) into the vascular system. As shown by a study of the arteries to the peripheral divisions of the facial nerve, each nerve division receives branches from arteries adjacent to its exit from the parotid gland, as well as along its course, and at its termination in the facial musculature. These arteries parallel the course of the nerve for a short distance, pierce and run in the epineurium and perineurium and end near, or anastomose with, the other arteries to that nerve. The source of these arteries varied with the arterial pattern of the face.

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EFFECTS OF HYPOTONIC SOLUTIONS UPON THE LIVING THYROID GLAND ¹

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SIX FIGURES

The thyroid gland of the living salamander, *Triturus torosus*, exhibits striking differences in its cytology when it is dissected from its fascial surroundings while in 100% and hypotonic Ringer solutions. The gland was exposed in Ringer solutions varying from 100% to 30%. Cytological observations were made at various intervals following the operation. In some cases the medium was changed to a different concentration a few hours after the operation, and any changes in the cells were noted. One thyroid in each of forty-five animals was studied.

MATERIAL AND METHODS

The Algire quartz rod apparatus, used for these experiments, has been described in an earlier report (Algire, '38, '39). Briefly it consists of a method for transillumination of the thyroid gland by means of a straight, tapering quartz rod. Observations may be made and photographs taken through the highest oil immersion systems of the compound microscope, while the gland, with its normal blood supply intact, is immersed in Ringer solution.

The salamander Ringer solution was slightly modified from the formula of Romeis ('22): Sodium chloride, 0.7 gm.; potassium chloride, 0.02 gm.; sodium bicarbonate, 0.02 gm.; calcium chloride, 0.02 gm.; double (or triple) distilled water, 100 cc.

¹ Aided by grants from the Bache Fund and the Bressler Alumni Research Fund.

Anaesthesia was accomplished by pithing the brain and the cranial end of the spinal cord. Heat was removed from the light source by Corning Aklo filters which absorb infra-red rays.

EXPERIMENTAL RESULTS

The appearance of the thyroid cells in 100% Ringer is taken as the standard condition. When viewed with the basal end of the cell in focus, the cell boundaries are sometimes clearly visible (fig. 1). The cells are pentagonal or hexagonal in shape and are almost completely filled by the nucleus. Around the nucleus is seen a thin strip of cytoplasm which is densely packed with plasma granules that appear motionless. The nuclear membrane is visible but not sharply defined. The contents of the nucleus appear lumpy, and it is usually difficult to distinguish any internal structures. These conditions remain unchanged as long as the animal's blood continues to circulate, usually a period of from 1 to 3 days.

An entirely different picture is seen in the thyroid of an animal which has been operated and observed in 30% hypo-

Fig. 1 Animal no. 474a34. Three and one-quarter hours after operation in 100% Ringer. Cells of thyroid are not swollen. No Brownian movement of plasma granules. Nuclei appear lumpy. Preparation unstained. $\times 720$.

Fig. 2 Animal no 474a34. Same field as in figure 1, $4\frac{1}{4}$ hours after operation and 1 hour after concentration of Ringer was changed from 100% to 30%. No cellular swelling and no Brownian movement of granules. Nuclei now clear. Preparation unstained. $\times 720$.

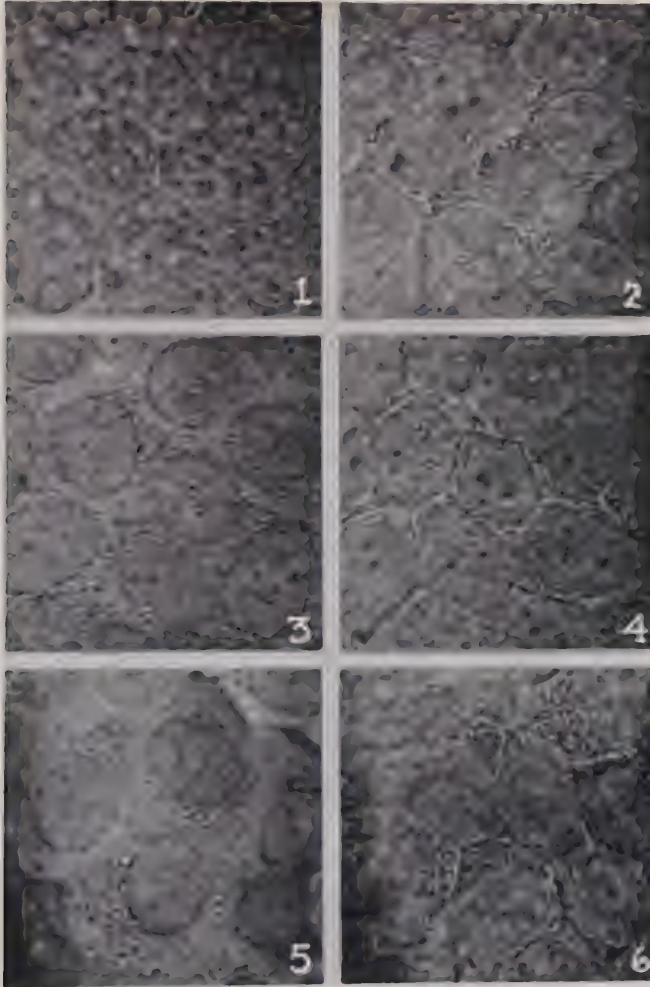
Fig. 3 Animal no. 474a37. Three and one-half hours after operation in 30% Ringer. Swollen cells indicated by increased distance between nuclei. Granules are in Brownian movement. Nuclei are clear. Preparation unstained. $\times 720$.

Fig. 4 Animal no. 474a37. Same field as in figure 3, $6\frac{1}{2}$ hours after operation in 30% Ringer. Spontaneous recovery is demonstrated. Cells have shrunk to standard size (nuclei now close together). All Brownian movement has stopped. Nuclei in this thyroid have remained clearer than usual. Preparation unstained. $\times 720$.

Fig. 5 Animal no. 474a8. Two and one-half hours after operation in 30% Ringer. Cells are greatly enlarged. Granules are in intense Brownian movement. Preparation unstained. $\times 720$.

Fig. 6 Animal no. 474a8. Same field as in figure 5, 28 hours after operation in 30% Ringer. Spontaneous recovery is demonstrated. In this specimen cells shrank to standard size and Brownian movement stopped by $4\frac{1}{2}$ hours after the operation. Nuclei appear lumpy. Preparation unstained. $\times 720$.

tonic solution (figs. 3 and 5). About 2 hours after the operation is begun, the gland is ready for observation under the compound microscope. The cells are greatly enlarged in size and are filled with a clear fluid cytoplasm. The granules, instead of being closely packed as in 100% Ringer, are widely separated and in intense Brownian movement. The great mobility of the granules indicates considerable dilution of the cyto-



Figures 1 to 6

plasm. The nucleus appears as a clear vesicle with sharply defined inclusions, surrounded by a distinct membrane. The nuclear inclusions are never seen moving. Only in a few cases was the nucleus sufficiently swollen so that it could be detected in the absence of special measurements, and the swelling was never in proportion to that of its own cell. The thyroid in 30% Ringer (fig. 5), when compared with the gland in 100% Ringer (fig. 1), shows considerable swelling of the cytoplasm, unaccompanied by nuclear swelling. In general all details of the cell are much more sharply defined in 30% than in 100% Ringer.

In six animals prepared in 100% and then changed to 30% Ringer for further observation, the cells did not swell (compare fig. 2 with figs. 3 and 5). Brownian movements are not observed. The nuclei, however, become much clearer, and structures within the nucleus become visible. The nuclear membrane, cell boundaries and plasma granules become more sharply outlined.

When animals are operated in 30% and then changed to 100% Ringer, Brownian movement stops 1 to 2 hours after the medium is changed, and the granules become arranged in a closely packed manner. Concurrently, the swollen cells become much smaller. The nuclei, which had previously been clear and sharply outlined, become lumpy in appearance, and their membranes are less distinct.

At first it was assumed that the replacement of the 30% Ringer by the 100% solution was responsible for the recovery of the cell. However, results from a series of twelve animals operated in 30% Ringer and allowed to remain in this concentration continuously, show a similar return to the standard condition. Three to 5 hours after the operation, spontaneous changes in the cell are clearly seen (figs. 4 and 6). Although continuously in 30% Ringer, the gland undergoes changes, all in the direction of a gradually increasing dehydration, and thus a return, from its hydrated condition, to its standard state. The Brownian movements become less and less pronounced and finally stop completely, and the granules again

become closely packed. The boundary of the nucleus becomes less distinct, while its contents gradually assume a more lumpy appearance. Simultaneously, the cells decrease in size. Vacuoles appear in most of the cells, and these vacuoles increase rapidly both in number and size. Six hours after operation, except for the presence of the vacuoles, the majority of the cells of the thyroid follicle have returned to the standard condition, that is, the state in which they are found when the operation and observations are carried out in 100% Ringer. The vacuoles reach maximum size by the time the cells have returned to their standard appearance. They then begin to

TABLE 1

Relation of concentration of Ringer at time of operation to occurrence of Brownian movement in thyroid cells.

CONCENTRATION OF RINGER (%)	NUMBER OF ANIMALS	
	Brownian movement	No Brownian movement
100	0	10
80	3	3
70	5	1
50	2	1 (?)
30	20	0

get smaller at a very slow rate, until they completely disappear.

In some cases the cells which undergo the most excessive swelling do not show the typical spontaneous recovery. Either they burst, or they shrink to standard size and Brownian movement stops, but their contents remain disorganized, and their nuclei are irregular in shape.

Operations were also carried out in 50%, 70% and 80% Ringer solutions. The degree of cellular swelling and of Brownian movement is found directly proportional to the amount of dilution (table 1). In all cases the spontaneous recovery takes place in the same way that it does in 30% Ringer. The nuclei appear equally clear in all concentrations between 30% and 80%.

In 30% Ringer cellular swelling and granular movement results no matter how slightly the gland is manipulated during the operation. On the other hand, in these experiments, excessive handling of the gland in 100% Ringer failed to elicit swelling or Brownian movement.

In order to determine whether the activated thyroid responds to hypotonic solutions in the same way as the unstimulated gland, a preliminary series of four animals was injected with anterior pituitary lobe extract for 3 days prior to operation. The thyroid cells exhibited typical signs of stimulation, but the response to 30% and 100% Ringer was the same as in uninjected animals.

Six of the animals whose thyroids showed the typical hydration and recovery, when in hypotonic solutions, were injected with either neutral red or Janus green. Although the plasma granules of these animals absorbed the vital stains, the nuclei remained completely unaffected. However, after death of the animals the nuclei became diffusely stained.

DISCUSSION

Cellular swelling after the lowering of the osmotic pressure of the outside medium is generally accepted as a fundamental property of protoplasm in isolated cells or tissues (McCutcheon and Lucké, '26). If the medium with which the cell is in osmotic equilibrium is diluted with distilled water, the cell swells as a result of diffusion of water into it, until it again reaches osmotic equilibrium with its surrounding solution.

Landis ('27) found that if the capillary wall were injured by mechanical pressure, its permeability to water was increased, and Lucké and McCutcheon ('30) reported that permanent injury may result in isolated cells after a prolonged exposure to hypotonic sea water. In the present experiment upon the entire living thyroid gland both factors are seen operating simultaneously. A hypotonic medium of only 30% was, in itself, incapable of causing swelling or Brownian movement, but, combined with the apparent mechanical injury of the operation, marked disturbances in permeability resulted.

Since no cellular swelling or Brownian movement was ever observed when the operation was carried out in 100% Ringer, it would appear as though the latter concentration offers a better protection against mechanical shock to the cell membrane than the dilute solution.

Lucké and McCutcheon ('26) found that swelling induced in *Arbacia* eggs by hypotonic solutions was reversible. When the cells were returned to normal sea water they shrank to their normal size, with no attending injury. The spontaneous shrinking of the swollen thyroid cells and their return to a standard appearance, while they remain continuously in a hypotonic medium, is not easily interpreted. The possibility of this phenomenon being a manifestation of permanent injury or death has been considered. The exceptional cases have been noted, in which the most excessively swollen cells did not recover their standard appearance, but either swelled until they burst or finally shrank and cytolyzed. Lucké and McCutcheon ('30) reported an observation of cellular swelling followed by shrinking in a hypotonic medium, for cells that were definitely injured by heating. From their study of the course of the swelling of dead cells in various isotonic solutions they concluded that if observations are extended over a sufficient length of time it is generally observed that swelling is followed by decrease in volume ('32).

In the present experiment, however, the cells not only became dehydrated but also assumed a well organized appearance. The dehydration was accompanied by rapid appearance of many vacuoles, which gradually disappeared. This observation suggests that the vacuole may play a part in regulating the water content of the cytoplasm. The granules stopped moving and became arranged in their typical closely packed manner. This standard and apparently healthy appearance usually remained unchanged as long as the animal lived, or a period of 3 days in some instances.

The nucleus of thyroid cells that were vitally stained after shrinking showed no increased affinity to the dyes, as is usually observed in dead cells (Lewis and McCoy, '22; Chambers, '23).

SUMMARY

A hypotonic medium increases the sensitivity of the thyroid cell to mechanical injury. Marked intracellular disturbances, consisting of an increase in permeability, swelling, and excessive Brownian movement of granules in the cytoplasm, occur in the cells of the thyroid gland of the living salamander if the gland is dissected from its fascial surroundings while in 30% Ringer solution, but not while in a 100% Ringer medium. If 100% Ringer is used during the operation, and is then replaced by the hypotonic solution, the cells remain essentially unaffected. Both factors—the hypotonic medium and the mechanical disturbances of the operation—must operate simultaneously to induce the changes.

Three to 6 hours after the cellular swelling and Brownian movement have been induced by the operation in hypotonic Ringer, the cells return spontaneously to their standard appearance, that is, to the condition associated with 100% Ringer.

ACKNOWLEDGMENT

The author is indebted to Dr. Eduard Uhlenhuth for suggesting this problem and for offering advice during the course of the experiments.

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OBSERVATIONS ON THE SO-CALLED "AZUROPHILIC GRANULATION" OF PROERYTHROBLASTS

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ONE FIGURE

Segerdahl ('35) described vacuole-like structures enclosing fine, granular, reddish staining material in the cytoplasm of the early megaloblasts (promegaloblasts). She believed the phenomenon to be an artefact probably caused by rupture of the nuclear membrane allowing the parachromatin (oxyphilic) to escape into the cytoplasm. Jones ('36) observed similar granules in the cytoplasm of promegaloblasts in one of several bone marrow preparations of Addisonian pernicious anemia submitted to him for morphologic studies. Although he noted that the granulation referred to in his paper as "azurophilic granulation" was neither well defined nor stained as intensely as the myeloid azure granules, he, nevertheless, felt that the granules were identical with those of the myeloblast. In his own series of biopsied human bone marrows, however, he was unable to detect the granulation either in promegaloblasts or pronormoblasts. Therefore, Jones felt that the granules "as a matter of fact, are quite rare in their appearance." He further stated that "It is not within the scope of this paper to discuss . . . as to whether or not the azurophilic granulation in dry imprints represents some different physico-chemical state of the cytoplasm. We do know that by utilizing the same technique throughout, i. e., the dry smear or dry imprint, all transitions exist between

¹ Parke, Davis and Company Fellow in Clinical Hematology.

the azurophilic granulation in the myeloblasts (lymphoidocyte) and that of the promegaloblasts.”

It is the purpose of this paper to point out that (a) the postulate of Jones “the azurophilic granulation substantiates the opinion that the promegaloblast is derived from the myeloblast because of the presence of transitions between azurophilic granulation of the myeloblast and that of promegaloblast” has become debatable during the past few years through the progress made in the field of colloid chemistry, (b) to present evidence that the Segerdahl-Jones granulation is apparently precipitated colloid protein, (c) that the substance is a constituent of the cytoplasm of the normal and pathologic proerythroblasts, and (d) recent staining studies carried out by the author showed that the so-called “azurophilic granulation” of proerythroblasts is different from the “azurophilic granulation” of the myeloblast.²

MATERIAL

The bone marrow for this study was obtained during life by means of the aspiration method. The sternum was punctured at the level of the second costal interspace. Twelve healthy males served as subjects. The marrow was obtained and stained according to the methods of Schleicher ('42). Because the literature contains various divergent descriptions of the morphology of the pronormoblasts, the cells are herewith redefined, especially since the observations were made entirely on this particular cell type. Two stages of the cytogenesis of the pronormoblast will be described and referred to in this paper as “early” and “late.” Intermediate types may then be easily recognized.

EARLY PRONORMOBLAST (FIG. 1-B).

The size of the cell ranges from 13.5 to 16 microns and thus has the same diameter or is slightly larger than the myeloblast. The nucleus occupies between two-thirds to three-fourths of the cell body, and contains from two to four

² Unpublished data.

or more irregular fairly well defined or confluent and frequently screened bluish tinted nucleoli. The chromatin strands are arranged in a "sieve like" pattern similar to that of the myeloblast, although the chromatin strands of the pronormoblast are slightly coarser, almost granular and stain darker. The chromatin is not prominently compressed about the nucleoli. The interchromatin spaces are larger and the oxyphilic parachromatin is more conspicuous than in the myeloblast. The nuclear membrane is not very distinct, although the nucleus is fairly well demarcated. The cytoplasm is not abundant and ranges in color from light to medium blue. Condensation of protoplasm at the periphery is prominent. Scattered throughout the slightly flaky cytoplasm are from one to several small, homogeneous areas not sharply defined, frequently barely perceptible, or patches of granular aggregates, both structures showing a conspicuous affinity for acid dyes. The granules are poorly defined in contrast to the sharply outlined azurophilic (reddish-blue) granules of the myeloblast. The cytoplasm frequently shows remnants of "cytoplasmic bridges" (pseudopods) which linked the cell with other components of the erythroid island, the structure of which will be published elsewhere. The cell does not contain hemoglobin. Intermediate forms between the rounded up reticulum cell (fig. 1-A) and the early pronormoblast (fig. 1-B) cannot be determined with certainty in the normal adult human bone marrow because of the extreme sparsity of heteroplastic proliferation.

LATE PRONORMOBLAST (FIG. 1-C).

The size of the cell ranges from 13 to 15 microns and thus is only slightly smaller than the early pronormoblast. The nucleus still shows the characteristic relationship to the cell body. It is round, reticular and has about the same number and type of nucleoli as the early form. The chromatin strands are thicker, more deeply staining and thus the oxyphilic parachromatin is more apparent than in the early pronormoblast.

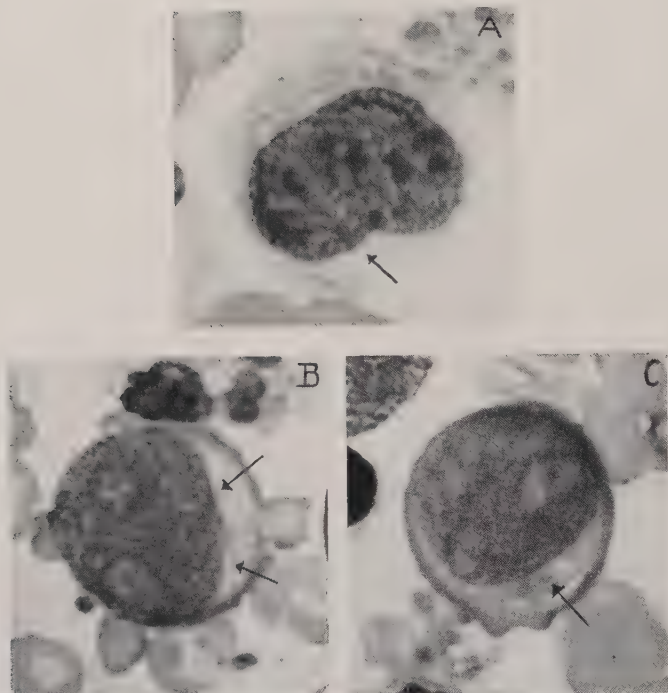


Fig. 1 Normal adult human bone marrow (sternum).

A. Rounded up reticulum cell showing an homogenous aggregate of colloid protein (oxyphilic) in the cytoplasm (at arrow).

B. Early pronormoblast showing finely precipitated colloid protein (oxyphilic) in the cytoplasm (at arrows). The large, round and reticular nucleus has two large, irregular, fairly well defined and two screened confluent bluish nucleoli. The parachromatin is prominent. The cytoplasm is slightly flaky, medium blue showing a conspicuous condensation at the periphery as well as remains of several cytoplasmic bridges (pseudopods) of irregular size and shape.

C. Late pronormoblast showing in a flaky cytoplasm a poorly defined perinuclear zone and "Hof." The latter contains coarsely precipitated colloid protein (oxyphilic) at arrow. The round, reticular nucleus shows the relationship to the cell body characteristic of the pronormoblast. The chromatin strands are thicker and stain slightly deeper than in the early pronormoblast. The oxyphilic parachromatin is more conspicuous. The character of the nucleoli is the same as in the early form. The cytoplasm, though, is more basophilic. Two remnants of cytoplasmic bridges near the arrow.

The nuclear membrane is distinct. The cytoplasm is conspicuously flaky and shows a slightly greater affinity for basic dyes. Condensation at the periphery is still prominent. A more or less well defined perinuclear zone and "Hof" contain either finely or coarsely precipitated oxyphilic protein. The granules are not sharply defined. Infrequently aggregates simulate myeloid azurophilic granules or form rod-like structures. The cytoplasm may or may not show remnants of cytoplasmic bridges. The cell does not contain hemoglobin. Mitotic figures are encountered in this cell type.

During an investigation of domestic blood stains for staining aspirated and imprinted human bone marrow, oxyphilic patches and granular structures were observed in the cytoplasm of pronormoblasts. The location of these structures corresponded to those reported by Jones for the so-called "azurophilic granules" in the cytoplasm of promegaloblasts. Careful staining experiments showed that the structures deviated conspicuously from the behavior of the azurophilic granules of the myeloblast. The impression was gained that the myeloid azurophilic granules and the oxyphilic granules in the cytoplasm of proerythroblasts had colloidal properties, although the two substances seemed to be different, that is, the granules appeared to be products of dissimilar colloidal systems.

Cells developing along different lines most likely have a dissimilar metabolism and thus distinct characteristic substances are being formed at certain developmental stages of each cell. Intricate complex chemical reactions going on within the protoplasm continuously influencing the physico-chemical state of the cell, bring about slight or profound modifications of the properties of the substances and colloid systems which the morphologist may observe in the cytoplasm for example, as homogeneous or granular structures. It is a well known fact that precipitation of a colloidal protein occurs according to whether the concentration of the protein is high or low, and whether the isoelectric point is reached slowly or quickly, although a homogeneus emulsion results when a colloidal

system is in a gelatinous state. Niethhardt ('35) observed that the isoelectric point of most albuminous substances is in the acid range and is shifted toward neutral during the drying process of the cell. This alteration of properties is a major factor in the production of artefacts so frequently encountered in cytology. The effect of rapid and delayed drying upon an albuminous substance, the "Cabot rings," and the resulting variability of affinity of the colloidal protein structures for acid and basic dyes have been demonstrated (Schleicher, '42).

The fact that the azurophilic granules within the cytoplasm of the myeloblast are still sharply defined and show the original hue when the granules in the cytoplasm of the proerythroblast have lost much, if not all, of their affinity for acid dyes, led to the recognition of differences in the nature of these granulations. Demonstration of the colloidal protein nature of the granulation of proerythroblasts furnished also the answer for their assumed "absence."

EXPERIMENT FOR DETERMINING THE NATURE OF THE GRANULATION

Ten smears were made from each of the aspirated bone marrows. The smears were rapidly whipped through the air to accelerate drying. Two preparations were immediately stained and the remaining ones stained in sets of two at intervals of 1, 2, 3, 12 and 24 hours. It was observed that in the preparations stained immediately, the greater percentage of pronormoblasts showed in their cytoplasm homogeneous patches, and some early and late pronormoblasts showed a fine granulation. Both structures stained uniformly oxyphilic. In the 2- and 3-hour specimens the structures were less conspicuous and varied in hue from pale to deep pink. In the 12-hour specimens the structures were practically absent. Occasionally a pronormoblast showed from one to several coarse, round or irregularly shaped structures which exhibited a conspicuous affinity for basic dyes or were tinted "reddish-blue." In the 24-hour preparations, a few pronormoblasts showed from one to sev-

eral faint, blue granules generally located at the periphery of the "Hof."

Thus it may be theorized that a profound alteration of the colloidal system occurs during the drying process which appears to be continuous as indicated by the type of precipitation and the affinity of the protein for acid and basic dyes. Eventually the affinity for either dye is lost and this phenomenon may well explain the absence of the structures in the cytoplasm of the proerythroblasts in the preparations examined by Jones. It is assumed that Jones adhered to a method practiced by European hematologists, i. e., to air dry bone marrow smears or imprints for 24-hours before applying blood stains.

DISCUSSION

With the increasing use of sternal marrow biopsies for diagnostic, prognostic and therapeutic purposes, the origin of the proerythroblasts in health and disease has become a major issue among morphologists. It would seem from the experiment described above that the granules in the cytoplasm of proerythroblasts are derived from a colloidal system different from that which forms the azurophilic granules of the myeloblast. The pronormoblast granulation seems to be the expression of a colloidal system changing from an emulsion or gelatinous state to a precipitative state. The protein appears to be amphoteric in nature. The protein is a normal constituent of the cytoplasm and time is an important factor for its demonstration. The protein has a strong affinity for acid dyes immediately after the cell has dried, although this property is lost with the shifting of its isoelectric point. Between 1 to 3 hours after dying, the protein exhibits a strong affinity for basic dyes, and this blending of the color of the protein in the latter phase with the basophilic color of the cytoplasm may be so complete that it is not possible to separate one from the other. This is especially true when the perinuclear zone or the "Hof" is not well demarcated. It is thus the time elapsed before the smeared or imprinted marrow is stained, rather than the rate of maturation of the cell as as-

sumed by Jones, that determines the oxyphilic and demonstrable phase of the protein, i. e., its presence or absence.

The "Hof" generally contains more protein than the perinuclear zone. It is assumed that this is due to a greater fluid state of the "Hof" which permits the protein to migrate into it during the drying process, or that there is normally a greater concentration of protein within this particular area.

The protein is not identical with the substance that forms the azurophilic granules of the myeloblast and, therefore, does not substantiate the opinion that the myeloblast is the precursor of the proerythroblast. Furthermore, the demonstration of the protein in the cytoplasm of the rounded up reticulum cell does not necessarily indicate that the latter is the precursor of the pronormoblast. The presence of the protein in the cytoplasm merely suggests that the cell has reached in its cytogenesis a certain physico-chemical phase. The various types of protein precipitates present in the cytoplasm of pronormoblasts undergoing mitosis are believed to be the result of a breakdown, redistribution or creation of colloidal systems.

The experiment revealed the reason for the assumed rare appearance of the granulation in the cytoplasm of proerythroblasts by bringing out the colloidal character of the protein and variability of its affinity for acid and basic dyes. It is of interest that the protein is not demonstrable in the basophilic normoblasts, the next developmental stage of pronormoblasts. It is believed that the observations reported here do not substantiate the opinion that the granules in the cytoplasm of proerythroblasts are "myeloid azurophilic granules."

SUMMARY

1. The granulation in the cytoplasm of proerythroblasts first described by Segerdahl in the promegaloblasts and identified by Jones as "azurophilic granulation" is most likely precipitated amphoteric colloidal protein.

2. The reason for the assumed "rare appearance" of the granulation is the variability of affinity for acid and basic

dyes by the protein particles. The amphoterism is seemingly the expression of a profound alteration of the colloidal protein system during the drying process.

3. Demonstration of the protein is dependent upon the time allowed for a smear or bone marrow imprint to air-dry.

4. The granules in the cytoplasm of proerythroblasts are derived from a different colloidal system than the azure granules of the myeloblast.

5. The so-called "azurophilic granulation" in the cytoplasm of proerythroblasts has no cytogenetic significance other than that the cells have reached a certain physico-chemical phase in their development.

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CULTIVATION OF THE EARLY CHICK EMBRYO IN VITRO

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ONE PLATE (FOUR FIGURES)

It has been the experience of McWhorter and Whipple ('12) that the cultivation of embryos in vitro prior to the 3- to 4-somite stage, or about the twenty-fourth hour of incubation, is not successful largely because of the difficulty of removing the blastoderm from the egg at such an early stage. On the other hand, the method of cultivation of the whole yolk in a glass dish, suggested by Vollmer ('35), would not meet the requirements of frequent handling. Similarly, the cultivation of the early blastoderm in a partially opened egg (Romanoff, '31), in spite of the fact that it is successful, and is suitable for photographic recordings (Romanoff, '38) permits neither handling nor close observation through the very thick layer of albuminous sac. Accordingly, it would seem that a method which not only meets the above requirements but is also simple, flexible and suitable for dealing with a large number of specimens, is much to be desired in modern embryological technic.

Such a need was felt acutely in our recent biophysical studies on the effect of x-rays (Romanoff and Bless, '42; Bless and Romanoff, '43) and of ultraviolet stimulation of the avian egg (Romanoff, '43). In these studies it was found necessary to carry on frequent measurements of the bioelectric potentials of the blastoderm from the fresh egg to the 24-hour stage. In order to insure the immediate contact of one of the electrodes with the blastoderm (Romanoff and Cottrell, '39)

the albuminous sac of the thick layer had to be ruptured and moved aside. A successful technic for physical measurement of the blastoderm, after rupturing the albuminous sac, was to drop the egg contents into a shallow glass dish, about 10 cm. in diameter, with a depression at the center for holding the yolk with the blastoderm in the uppermost position.

After this rough treatment all attempts to cultivate the egg in a beaker with the blastoderm in the uppermost position (suitable for observation) failed. It was evident that the yolk without the protective albuminous coating has a tendency to float up on the surface thus exposing the blastoderm to the air. Under such conditions the development of the embryo was impossible because of rapid surface evaporation. Therefore, after the experimental test it was found that the immediate submersion of the yolk into albumen was essential. Also several other special precautions were found to be necessary in order to maintain normal development of the embryo outside the shell for a reasonable length of time.

Consequently to meet all these requirements a special method was devised for the cultivation of embryos outside the shell. The use of standard glassware with simple additional arrangements seemed most desirable for the manipulation of a large number of specimens.

In this method it was found that the low form 50 cc. beaker is most satisfactory to hold the average contents of the chicken egg. The yolk is kept submerged in the albumen with the blastoderm uppermost by means of a heavy glass ring about 6 mm. thick and 20 mm. in inside diameter supported on three legs 30 mm. long (fig. 1). After the egg is in position it is covered by an inverted 150-cc. beaker of low form and placed in an incubator (fig. 2). The use of a glass-topped incubator permitted us to make a continuous observation of embryo development without disturbing the specimens.

In addition some special requirements were met for the successful cultivation of the embryo. Some of these already have been worked out for those cultivated *in vivo* (Romanoff,

'31, '33). First, the excessive evaporation of egg contents must be prevented; and second the egg must have access to fresh air. It was found that the selection of the proper size of the inverted beaker, and the use of the still-air type of incubator with an elevated wire screen floor, will assure a sufficient supply of fresh air in the whole assembly without the rapid evaporation of egg contents. It was also determined experimentally that with a temperature at the level of the blastoderm of about 37.5°C . and a surrounding relative humidity of 60%, uninterrupted development can be attained up to the 48-hour stage (figs. 3 and 4). Frequently the development may proceed normally beyond this stage, but the field of observation will be partially obstructed by the walls of the glass ring.

It is of interest to note that the method may be useful not only when there is a necessity for frequent handling and continuous observation of the developing blastoderm, but also that it has been possible to use it — by controlling the depth of submersion of the blastoderm in the albumen — in numerous studies of the influence of radiation, drugs and various chemical preparations on early development of the embryo. Besides, even after the fresh eggs have been opened and used for various physical measurements, the further cultivation by this method may offer an excellent test of fertility.

SUMMARY

The method and apparatus described is simple and provides for easy handling of specimens cultivated outside the shell up to 48-hour stage.

After the rupture of albuminous sac the yolk is submerged by means of a heavy glass ring into the albumen.

The container is covered with a suitable beaker to prevent an excessive evaporation of egg material, and placed on the elevated wire-mesh floor of the incubator to assure the necessary supply of air.

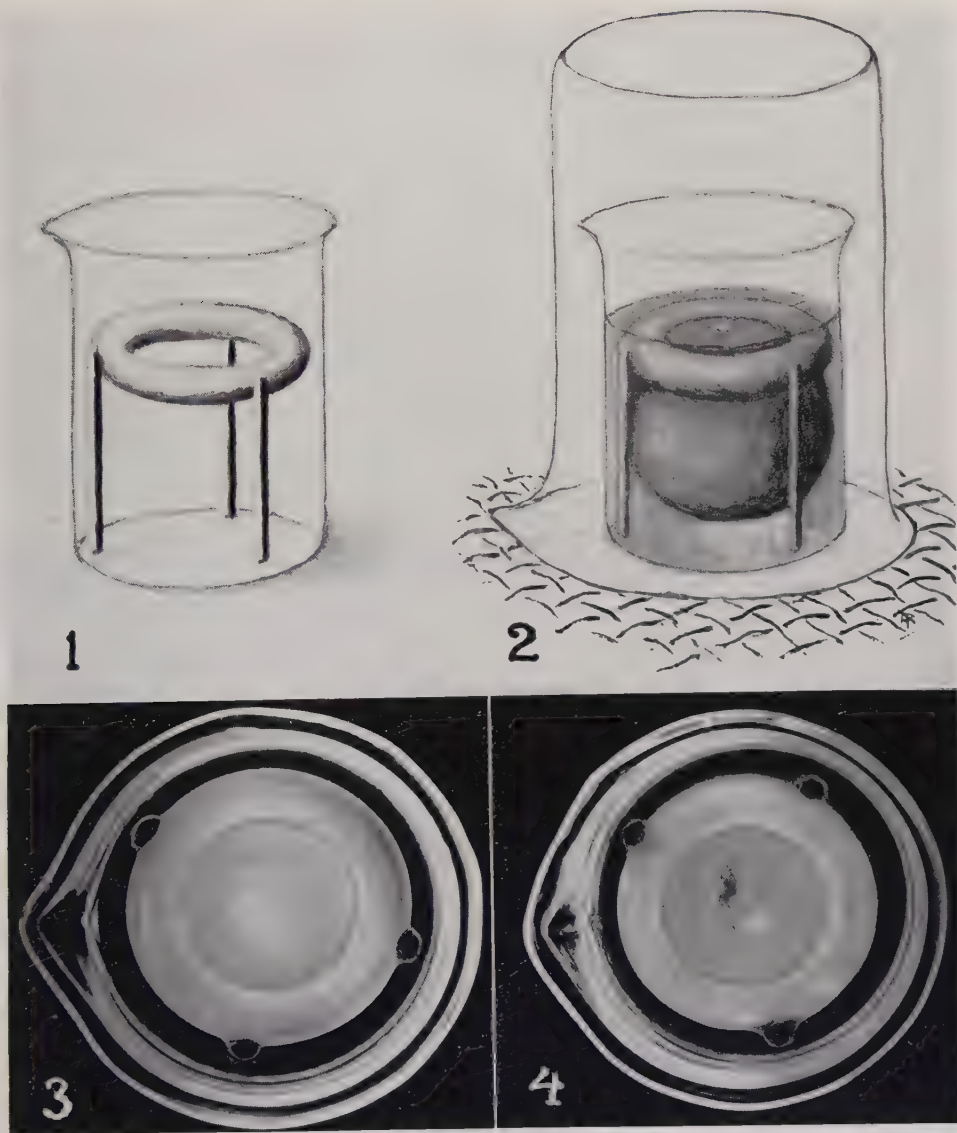
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PLATE 1

EXPLANATION OF FIGURES

- 1 The container for a chicken egg consisting of the low form 50-cc. beaker with a heavy glass ring supported by legs.
- 2 An assemblage of the apparatus showing the yolk submerged in the albumen with the blastoderm in the uppermost position and the egg container covered by an inverted 150-cc. beaker of low form.
- 3 and 4 Photographs of the cultivated embryos at two stages of development (about 16 and 36 hour).



WEIGHTS OF INTERRENAL GLANDS OF ELASMOBRANCHS

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ONE FIGURE

The size or weight of a given healthy endocrine gland is an indication of its potential capacity for secretion.

Adrenal weights have been determined in many species of birds and mammals and in forms as low as the reptiles. In lower vertebrates the homologous tissues are often so scattered that it is difficult to find and dissect them. In the elasmobranchs, interrenal tissue can be dissected so that accurate weights are obtainable. Thus it is possible to determine the weight of the cortical homologue in one of the lowest of the fishes. In no other vertebrate can the cortex or its homologue be directly weighed.

The interrenal tissue of elasmobranchs is easily distinguished by its yellowish-white color. In the selachians, the interrenal is a lobulated rod which may be broken into pieces anteriorly. It lies embedded in the posterior part of the kidney along the midline. In the skates the interrenal bodies are found on one or both sides of the midline, lying ventromedially but not embedded in the kidneys. Small interrenal bodies are occasionally found well removed from the main masses of tissue.

We have weighed the interrenal tissue of representatives of ten species of elasmobranchs in 135 specimens. These specimens were obtained in the process of regular fishing operations near Woods Hole from June to September.

Interrenal tissue was carefully separated from other tissue and then weighed to the nearest milligram on either a Roller-

Smith torsion balance or a quantitative chemical balance. In the latter instance it was enclosed in a small vial, tightly stoppered. Total body weights were obtained by means of a Chatillon spring balance to the nearest 25 gm. (with the exception of *Raja stabuliforis* which was weighed to the nearest 100 gm.).

TABLE 1

Arithmetic mean body weights and interrenal weights with standard errors for ten species of elasmobranchs.

FAMILY AND SPECIES	SEX AND NUMBER	BODY WEIGHT ARITHMETIC MEAN, GM.	INTERRENAL ARITHMETIC MEAN, GM.	WEIGHT PER CENT BODY WT.
<i>GALEIDAE</i>				
<i>Mustelis canis</i>	9 males	2,564 $\pm$ 209.4	0.243 $\pm$ 0.0339	0.0092
(Smooth dogfish)	6 females	2,933 $\pm$ 626.0	0.194 $\pm$ 0.0437	0.0066
<i>Prionace glauca</i>				
(Blue shark)	1 male	48,640	1.040	0.0002
	1 female	27,000	0.760	0.0003
<i>Carcharhinus obscurus</i>	1 female	13,100	0.125	0.0010
(Dusky ground shark)	1 undet.	19,540	0.437	0.0022
<i>SQUALIDAE</i>				
<i>Squalus acanthias</i>	20 females	2,987 $\pm$ 151.3	0.171 $\pm$ 0.0144	0.0057
(Spined dogfish)	(with embryos)			
<i>RAJIDAE</i>				
<i>Raja stabuliforis</i>	1 male	15,200	0.610	0.0040
(Barn-door skate)	11 females	11,206	0.428	0.0038
<i>Raja diaphanes</i>	1 male	530	0.019	0.0035
(Big skate)	1 female	1,050	0.046	0.0047
<i>Raja eglanteria</i>				
(Clear-nosed skate)	4 females	2,390	0.053	0.0023
<i>Raja erinacea</i>	17 males	562 $\pm$ 9.6	0.023 $\pm$ 0.0125	0.0036
(Common skate)	56 females	532 $\pm$ 9.5	0.018 $\pm$ 0.0012	0.0034
<i>TORPEDINIDAE</i>				
<i>Narcacion nobiliana</i>	2 females	39,600	1.130	0.0029
		14,000	0.396	0.0028
(Torpedo)	1 undet.	8,070	0.254	0.0032
<i>DASYATIDAE</i>				
<i>Dasyatis centrura</i>	2 males ¹	57,700	0.274	0.0005
(Sting ray)		40,000	0.181	0.0004

¹ Failed to find interrenals in four other specimens.

OBSERVATIONS

The average interrenal weights and body weights with sexes separate are shown in table 1, along with the interrenal weights per 100 gm. of body weight, which may be used for preliminary comparisons between genera and species. Some genera are represented by only a few specimens; comparisons involving these are therefore suggestive only. *Mustelis* and *Squalus* appeared to have relatively heavier interrenals than did *Raja* and *Narcacion*, while the interrenals in *Dasyatis* were exceptionally small. Indeed with the most careful dissection we failed to find the interrenals in four specimens of *Dasyatis*. All but one specimen of *Squalus* contained embryos. However, the relative weight of the embryos was small. A typical specimen contained four embryos weighing 30 gm. each, while the mother weighed 6630 gm. In some, the embryos were smaller. In *Raja* there were one or two interrenals. Of seventy-one specimens of *R. erinacea* in which the distribution was recorded, interrenals were found on both sides in forty-five, on the left side only in twenty-five, and on the right side only in one.

SPECIES DIFFERENCES

Uniform records were available for a large enough number of females of three species (*Raja erinacea*, *Squalus acanthias*, *Mustelis canis*) and of males of two species (*R. erinacea*, *M. canis*) to permit a further examination of species and sex differences in the amount of interrenal tissue. It is obvious that any comparisons between species or between sexes in amount of interrenal tissue should be made independently of differences in body weight. Particularly this will be desirable when there is a significant, but not necessarily a perfect, correlation between interrenal weight and body weight as there is in this case. For females, $r = 0.263$, which for 78 degrees of freedom has a probability of 2% of arising through chance alone, and for males, $r = 0.588$, which for $df = 24$ gives $P = 1\%$. The comparison may be accomplished by adjusting the interrenal weights to new values expected if all

individuals were uniform in weight. The method of performing this adjustment is that of a covariance correction (Fisher, '38). The analysis of species differences was performed separately for females and males, and of sex differences in *R. erinacea* only. The numbers of males and females in *R. erinacea* and *M. canis* were so markedly disproportionate that it was decided to attempt no complete analysis of a possible sex-species interaction.

Females. It became apparent when the data were tabulated on a scatter diagram that an increase in arithmetic mean body weight from one species to another was accompanied by increased variability about the mean. The same was true for interrenal weights. In order to compensate, at least partially, for this relationship between the means and variances, the scale was transformed from arithmetic to geometric, and all subsequent computations were performed with logarithms.

TABLE 2

Geometric mean body weights and gland weights of females of three species of elasmobranchs.

SPECIES	NUMBER	GEOMETRIC MEAN BODY WEIGHT, GM.	GEOMETRIC MEAN GLAND WEIGHT, GM.	ADJUSTED GEOM. MEAN GLAND WT.
<i>R. erinacea</i>	56	527.8	0.0153	0.0213
<i>S. acanthias</i>	19	2837.5	0.1590	0.0736
<i>M. canis</i>	6	2535.0	0.1630	0.0812
All species	81	879.5	0.0315	0.0315

Table 2 gives the geometric mean body weights and interrenal weights of the females of three species, two specimens of *S. acanthias* having been omitted because they were far outside the range of the remainder. In the final column of the table are given the adjusted geometric mean gland weights. The appropriate test of significance (F-test) indicates that these weights are significantly different as a set ($P = .05 - .01$). Further it is found that the dogfishes (*M. canis* and *S. acanthias*) are not significantly different from each other, but possess interrenal glands that are significantly larger than the glands of skates.

These relationships are portrayed graphically in figure 1. Each species with its geometric mean body weight and gland weight is plotted separately, along with the best fitting

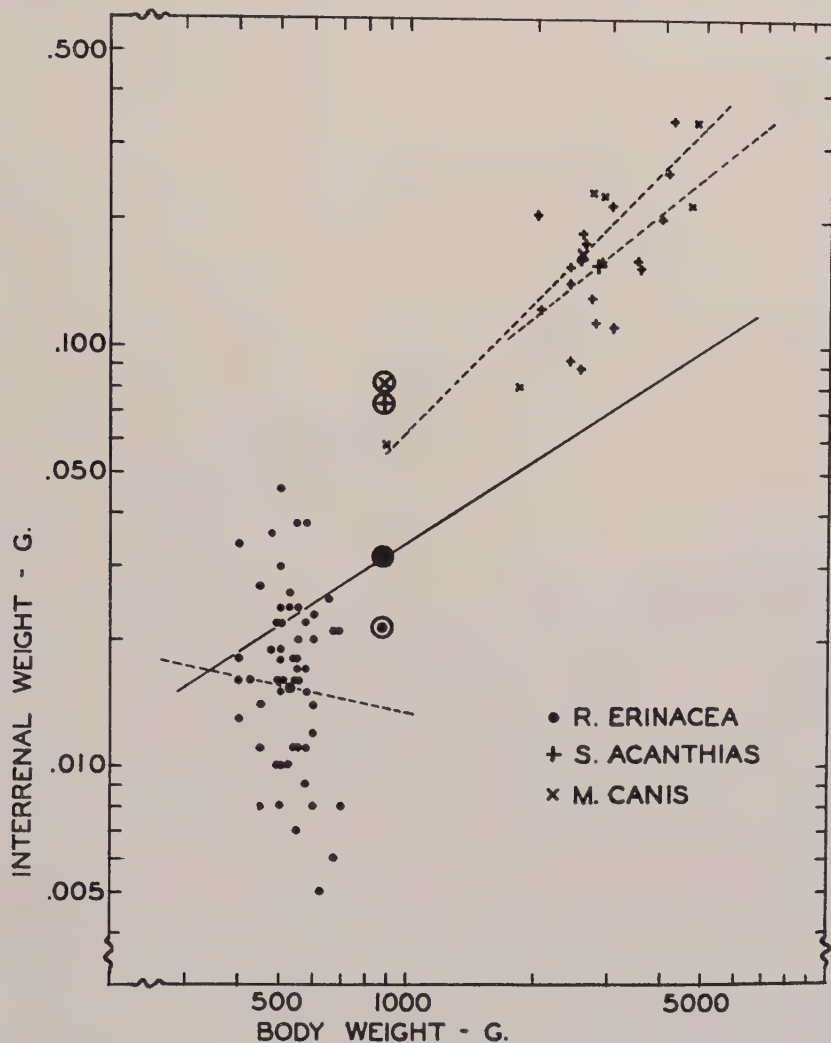


Fig. 1 Scatter diagram on logarithmic grid of the body weights and interrenal weights of females of three species of elasmobranchs. The broken lines drawn through the means show the relationship of gland and body weight for each species taken singly. The solid line drawn through the grand mean shows the general relationship based upon all species. The symbols enclosed in circles represent the adjusted mean gland weight for constant body size of each species.

straight line (broken line) that shows the relationship of gland weight to body weight within each species taken singly. In addition there is represented the generalized "within species" regression line. The removal of the effect of body weight differences is equivalent to shifting the gland weight means along lines parallel to the generalized regression line to the points indicated in the figure. These points we have found do not comprise a homogeneous set, the *R. erinacea* mean weight being significantly less than the others.

Skates are much less active than dogfishes but whether interrenal size bears any relation to activity is mere conjecture.

TABLE 3

Geometric mean body weights and gland weights of males of two species of elasmobranchs.

SPECIES	NUMBER	GEOMETRIC MEAN BODY WEIGHT, GM.	GEOMETRIC MEAN GLAND WEIGHT, GM.	ADJUSTED GEOM. MEAN GLAND WT.
<i>R. erinacea</i>	17	560.0	0.0208	0 0 35
<i>M. canis</i>	9	2465.5	0.2223	0.0 51
All species	26	935.5	0.0472	0.0472

Males. The analysis of the gland weights of males, performed in a similar fashion, gives slightly different results. These are shown in table 3. The skates still have a lower gland weight, but the difference could easily have arisen by chance considering the small size and large variability in the present samples.

SEX DIFFERENCES

In the single species, *R. erinacea*, in which the test could be reliably performed, there was no significant difference between the geometric mean interrenal gland weights of males and females. Actually the mean gland weight for females was 0.0153 gm. and for males, 0.0208 gm. The difference is even less after a trivial adjustment for slight difference in body weight of males and females.

DISCUSSION

It will be noted that the various species of Rajidae do not differ widely in their proportionate interrenal weights. The few individuals of Torpedinidae come within this range. The spined and smooth dogfish appear to have relatively the largest interrenals of the elasmobranchs. They are both much more active than the skates and torpedo.

It is noteworthy that the sharks studied contained relatively some of the smallest interrenals. But it is especially striking that the sting ray not only possessed small interrenals but that we failed to find these organs in four out of six. The whole region in which interrenal tissue might be found was carefully dissected. The contrasting yellowish-white color makes interrenal tissue easily distinguished, yet none was found.

A comparison of elasmobranch interrenal with mammalian cortex may be made. The percentage weight of cortex to the body weight in the latter can be calculated from the work of Elliott and Tuckett ('06) who determined the relative proportion of cortex to medulla. According to them, it is 5:1 in the dog, 20:1 in the rat and 62.5:1 in the guinea pig. Sato ('30) found that the mean weight of both adrenals, in percent body weight, in dogs was 0.014 for females. According to Donaldson ('24) the mean weight of both adrenals, in percent body weight, for a 200 gm. female albino rat was 0.026 while according to Bessessen and Carlson ('23) it was 0.05 in the guinea pig. On this basis, the percentage weight of the cortex to the body weight would be as follows: dog, 0.011; rat, 0.0247; and guinea pig, 0.049.

The largest elasmobranch interrenal, 0.009% (*Mustelis canis*), relatively is not far from the smallest mammalian cortex (dog). However the smallest elasmobranch interrenal (*Prionace*, 0.0003%, and *Dasyatis*, 0.0005%) is considerably smaller than this.

SUMMARY

1. Body weights and interrenal gland weights were obtained for 135 specimens of ten species of elasmobranchs with a view toward comparing the relative size of the interrenal

tissue within these species, and between these and other species.

2. In terms of weight of gland per 100 gm. body weight, it appears that interrenal glands of the elasmobranchs as a group are smaller than the homologue in such mammals as the dog and rat.

3. There are significant positive correlations between body weight and interrenal within the females of three species (*Raja erinacea*, *Squalus acanthias*, *Mustelis canis*) and within the males of two species (*R. erinacea*, *M. canis*) for which enough specimens were recorded to make the computations meaningful. The existence of these significant, but not perfect, correlations requires that comparisons between species be made upon some basis other than percentage body weight. The appropriate adjustment of the interrenal weights to a common basis independent of body size was made by a covariance correction.

4. For females only, the adjusted mean gland weight of *R. erinacea* is significantly smaller than those for *S. acanthias* and *M. canis*.

5. For males only, the adjusted mean gland weights of *R. erinacea* and *M. canis* are not significantly different.

6. For males and females of *R. erinacea*, there is no significant difference in gland weight.

7. Although the largest elasmobranch interrenal is not far, relatively, from the smallest mammalian cortex, the smallest elasmobranch interrenal is much smaller.

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DEVELOPMENT OF THE INNERVATION PATTERN IN THE LIMB BUD OF THE FROG

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FIFTEEN TEXT FIGURES AND TWO PLATES (TEN FIGURES)

INTRODUCTION

It is surprising that the normal development of nerves in the limb of the frog should have received so little attention, especially as the tadpole has been used repeatedly since the beginning of the century for experimental studies on innervation.

Before undertaking a proposed experimental project, it was found necessary to make this detailed study of the process of nerve pattern formation by examination of its progress at various stages in the ontogeny of larval frogs.

Since the factors determining pattern formation are intrinsic to the limb itself (Harrison, '35), it has been supposed that the limb components (skin, muscles, blood vessels) act somehow, either through chemical attraction, local electrical potentials, or by mechanical guidance, to fashion the invading nerve fibers into the typical pattern. However, no critical evidence could be obtained to show that any type of agent was adequate to direct nerve outgrowth or to produce a pattern.

A renewal of investigation on the problem of normal pattern development seems especially appropriate since recent studies by Weiss ('33, '34, '41) have brought to light some of the laws which govern the oriented outgrowth of nerve fibers in tissue culture and in deplantation experiments. It is of

¹ This research was done under the direction of Dr. Paul Weiss in partial fulfillment of the requirements for the degree of Doctor of Philosophy. It has been aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

particular interest to determine whether in the normal embryonic processes there is evidence of the action of these factors and whether they seem adequate to account for the establishment of the typical nerve pattern in the limb.

The anuran larvae are especially suited for the purpose of such a study since in these the hind limbs appear much later in ontogeny than in other vertebrates, including the urodeles. As a consequence it is possible to deal with an essentially embryonic limb in the larval form in which the ganglia and cord are relatively far advanced in development, thus permitting of a much more localized and precise operation than can be performed on the much younger stages of urodeles or other vertebrates.

METHODS

The present studies on limb nerve development were carried out on larvae of *Rana pipiens*, which had been reared from eggs in the laboratory under optimal conditions of feeding and temperature. These larvae were carefully staged and then fixed in either Bouin's or Zenker's fluid. Tissues were imbedded in paraffin and sectioned at a thickness of 8 micra. Heidenhain's modification of Mallory's azan triple stain was used for cytological elements, and Bodian's activated protargol-reduced silver impregnation for the neurofibrils.

Besides direct microscopic study of the sections, two- and three-dimensional reconstructions of the nerve pattern were made with the aid of a Bausch and Lomb microprojector. Two-dimensional reconstructions consisted of superimposed drawings of the sections involved. To study the complete limb bud innervation, three-dimensional reconstructions were made by tracing onto 6 by 12 inch glass plates drawings of each section made with the use of the projector. These plates were then assembled in order and cemented together with a plastic (isobutyl methacrylate Polymer) dissolved in xylol. Only the limb bud outlines and the nerve fibers were traced on the plates so that the whole nerve pattern could be seen in relief, permitting a clear visualization of spatial relationships.

Stereoscopic photographs of such reconstructions present the limb and its innervation in their true perspective (figs. 24 and 25).

STAGING

It became desirable in working with frog larvae to be able to refer conveniently to the level in the progress of development which a given animal had attained at a given time. The relation between age, or size, and progress of differentiation fluctuates too much to serve adequately as criterion for staging, especially during the very rapid changes which precede and accompany metamorphosis. A selection was, therefore, made of certain characters correlating better with general development by which the larval period could be divided into a convenient number of stages. For the choice of these characters a study of a statistically significant number of tadpoles has been made and will be published elsewhere. However, for convenience in this paper, outstanding external features by which some of the postembryonic stages may be recognized are listed below. These stages will be designated as "L" (i.e., larval) stages. They follow directly upon the twenty-five stages established by Shumway ('40, '42) for embryos of *Rana pipiens* which will hereinafter be referred to as "E" (i.e., embryonic) stages.

The average body length indicated for each stage is based upon measurement from snout to tip of tail made on larvae kept at a constant temperature of $20 \pm 1^\circ\text{C}$., in individual dishes, and maximally fed upon boiled lettuce.

Photographs showing the limb bud at some of the following stages are given in figures 16-23.

Stage L 1 — Oral suckers have disappeared. Four rows of labial teeth are present. Embryonic pigmentation has been replaced by larval pigmentation in chromatophores. Average body length, 12.5 mm.

Stage L 2 — Length of limb bud equal to one-half of its diameter. Average body length, 16.5 mm.

Stage L 3 — Length of limb bud equal to its greatest diameter. Bud constricted at its base. Average body length, 23 mm.

Stage L 4—Length of limb bud equal to one and one-half times its diameter. No bend in limb bud. Average body length, 34 mm.

Stage L 5—Length of limb bud approximately two times the diameter. Distal half of limb bud bent in ventral direction. No paddle flattening of future foot. Average body length, 40 mm.

Stage L 6—Medio-lateral paddle flattening of distal portion of limb bud. No indication of interdigital notches. Average body length, 43 mm.

Stage L 7—More pronounced paddle flattening. Fifth toe prominence separated from fourth by slight indentation of the paddle margin. Average body length, 50 mm.

Stage L 9—Interdigital notches 5-4, 4-3, and 3-2 present. First independent leg movements. Average body length, 56 mm.

Stage L 12—Margin of the lateral half of the 5-4 webb coincides with a line passing from the tip of first toe to tip of fifth toe. Average body length, 68 mm.

Stage L 18—All toe pads of foot are present. Leg and foot used in swimming. Ventral tail fin in region of cloaca reduced. Skin window for foreleg not yet present. Average body length, 71 mm.

Stage L 20—Forelegs freed from skin covering. Average body length, 41 mm.

DEVELOPMENTAL CHANGES BEARING ON INNERVATION

Stage L 1

Just before stage L 1, the hind limb bud is visible externally with the aid of a dissecting microscope as a very slight elevation in the groove between the lateral body wall and the base of the tail. Histological sections reveal that, at this time, some internal organization is already present. Figure 1 shows a frontal section through the limb bud of a larva in stage L 1. The appearance of the limb bud at this stage agrees with the description given by Filatow ('30, '33). The epidermis forming the dome of the bud swelling is distinctly different in appearance from that of the body wall and tail skin, the nuclei

of the inner layer of cells being larger, more spherical and much more closely packed together. That there is also a chemical difference is indicated by a marked color differentiation in the gold-toned silver impregnations, the epidermis of the limb bud being bluish, while elsewhere over the body it appears in purple or red.

Also noteworthy is the specialization of the somatopleural cells lining the portion of the coelom directly opposite the limb disc (ss, fig. 1). Unlike the extremely flattened squamous

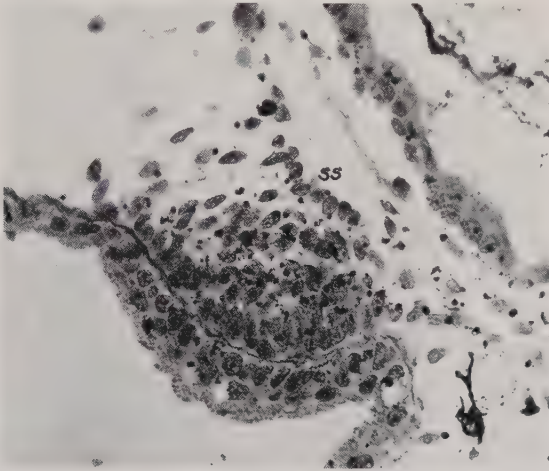


Fig. 1 Frontal section through the limb bud of a stage L 1 larva. ss, specialized portion of somatopleure. Mallory's azan triple stain. $\times 420$.

epithelium cells which form the coelomic lining elsewhere, those of this region are rounded and quite closely packed together, having lost their strict alignment. Occupying the space between the epidermis and the somatopleure, there is a mass of mesenchyme cells, densely packed near the epithelium of the bud and already showing a looser arrangement in the region bordering on the somatopleure. This latter looser portion, which becomes more prominent in the two succeeding stages, forms a connection between the limb base and the somatopleure and will be referred to as the "mesenchyme bridge".

The crowding of cells in the somatopleure, the radiating lines of the mesostroma and the elongated shape of the bridge cells all accord with Filatow's contention that the mesenchyme of the limb is being built up by immigration of cells from the somatopleure.

The spinal nerves 8, 9, and 10 approach the bud from a mediodorsal direction and lie between the somatopleure and the myotomes. They consist of two distinct types of fibers, a small number of relatively coarse fibers and a bundle of comparatively faint fibers, so fine as to resemble in size the fibrillae within a large axon. All the fibers of the first category can be traced easily through the serial sections. Some are seen leaving the main trunks at intervals to penetrate between the myotomes and become distributed through the already well differentiated body musculature. Only a very few of these larger fibers reach the level of the tail base where they end in the skin.

The fine fibers, which form a separate bundle lying adjacent to the heavier ones, taper peripherally, becoming finer and fainter until, at the level of the limb bud, they become invisible even under highest magnification.

It must be borne in mind that what appears in silver preparations to be the end of a growing fiber tip need not be considered its actual termination. Beyond the visible end, the fiber often extends in very fine pseudopodia which either fail to be sufficiently impregnated or are below the limit of microscopic visibility.

A few fibers of larger diameter and taking a heavier impregnation enter the epidermis of the limb bud at its median border (not visible in fig. 1), having come from a branch of the eleventh spinal nerve. This is the beginning of an intra-epidermal innervation which will be mentioned later.

Stage L 2

The visible changes occurring during the interval between the first and second larval stages are chiefly those of size and proportion. Accompanying these growth changes, certain positional shifts are noticeable by stage L 2 which materially affect

the development of the limb. To illustrate these shifts, diagrammatic frontal sections through animals at stages E 25 and L 2 are shown in figure 2.

Comparison of the two diagrams makes apparent the following changes: (1) That portion of the mesenchyme anchoring the limb bud to the somatopleure, which has already been designated the bridge (b), has been considerably increased in extent. (2) The axis of the limb bud (a-a) has shifted from

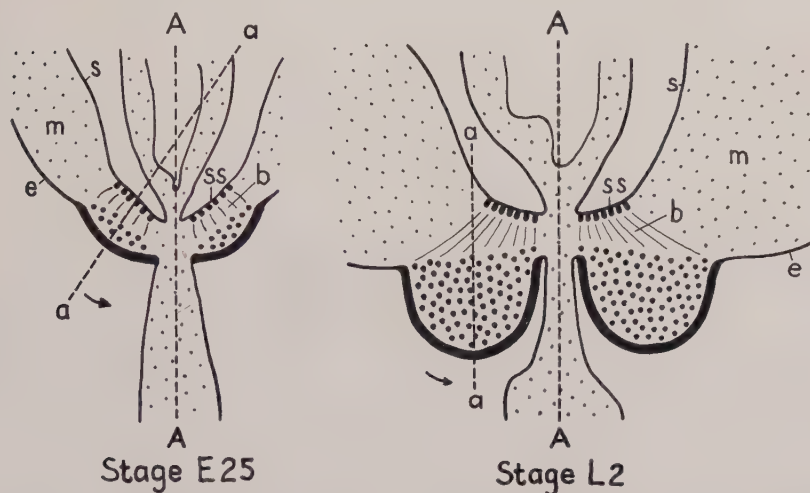


Fig. 2 Diagrammatic frontal sections through the limb buds of larvae in stages E 25 and L 2 to illustrate the positional shift of the bud occurring during this interval. A-A, body axis of the larva. a-a, axis of the limb bud. b, region of the bridge mesenchyme. e, skin. m, subdermal mesenchyme. s, somatopleure lining the coelom. ss, specialized portion of the somatopleure.

its oblique position of the earlier stage to one almost parallel with the body axis (A-A). This shift results in the transposition of the original anterior and posterior margins of the limb disc in stage E 25 to the respective lateral and medial surfaces of the bud in stage L 2. (3) During this shift the distance between the lateral border of the limb bud and the specialized portion of the somatopleura (ss) increases much more than the distance between the latter and the mesial border. In consequence, the bridge mesenchyme, being anchored to both the

limb bud and the somatopleura, shows much greater stretch in its lateral portion than elsewhere.

These changes in the limb bud occur in consequence of developmental happenings in other parts of the body. Filatow ('33) observed that during this interval there is a swelling of the intercellular matrix of the mesenchyme (m) found under the skin, causing an increasingly greater separation of the skin from the somatopleure. A tension is thus exerted upon the stroma of the bridge which extends between these two tissues, accounting in part for its rapid increase in extent. Coincident with this swelling is the establishment of the intestinal spiral, which further distends the body of the larva in front of the limbs and exerts a lateral pull on the limb bud. These forces, together with the rapid increase in diameter of the limb bud disc itself, are responsible for its lateral displacement and the shift of its axis, just described.

The stresses created by these shifts finds striking expression in the fine structure of the mesenchyme bridge at stage L 2 (fig. 3). The elongated shape and parallel arrangement of the nuclei, and the definite orientation of the mesostroma of the bridge (b), indicate clearly the direction of the tensional forces and their relative intensity at different points. That stresses resulting from organ growth may have such moulding effects on the surrounding mesenchyme has been emphasized by Weiss ('33, '39).

The nuclei of the mesenchyme within the bud are not evenly distributed. A layer of more densely packed cells (c) has formed next to the epidermis, which is thickest at its proximal dorsomedian region, opposite the point of entry of the nerves.

Many fine nerve fibers can now be traced into the mesenchyme of the bridge (nf) where they are seen to extend in the direction of the limb bud. After entering the bridge, the fibers of the three contributing spinal nerves have intermingled in such a way that any branches which may later emerge from them could contain fibers from all three of the lumbo-sacral segments. This region of fiber commingling is the future sciatic

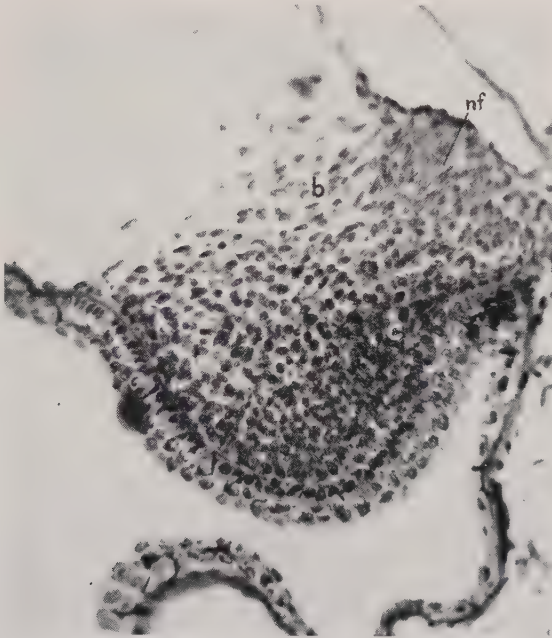


Fig. 3 Frontal section through the limb bud at a stage L 2 larva. Impregnated with silver according to Bodian and counterstained with azan. $\times 200$. b, mesenchyme of the bridge. c, condensation of limb bud mesenchyme. nf, nerve fibers.

plexus. It should be noted that at the time of its formation it is near the level of the base of the limb.

Stage L 3

From the beginning of its development, the limb bud has had a well differentiated epidermis consisting of two cell layers resting on a distinct collagenous basement membrane. At stage L 2, the outer layer has lost its squamous appearance so that now both layers have large spherical nuclei which lie close together. By stage L 3 (fig. 4), the outer layer around the basal part of the bud is flattening somewhat, while over the distal portion, both layers retain a more embryonic appearance.

Further evidence of the anchorage of the limb bud to the somatopleure by the mesenchyme of the bridge is seen here

in the constriction of the bud at its base, so that by stage L 3, it is no longer greatest in diameter at this level.

The distribution of mesenchyme within the bud at stage L 3 is, in general, the same as during the preceding stage. Although, on the whole, the cells are quite evenly distributed, the peripheral condensation seen in stage L 2 is still present,

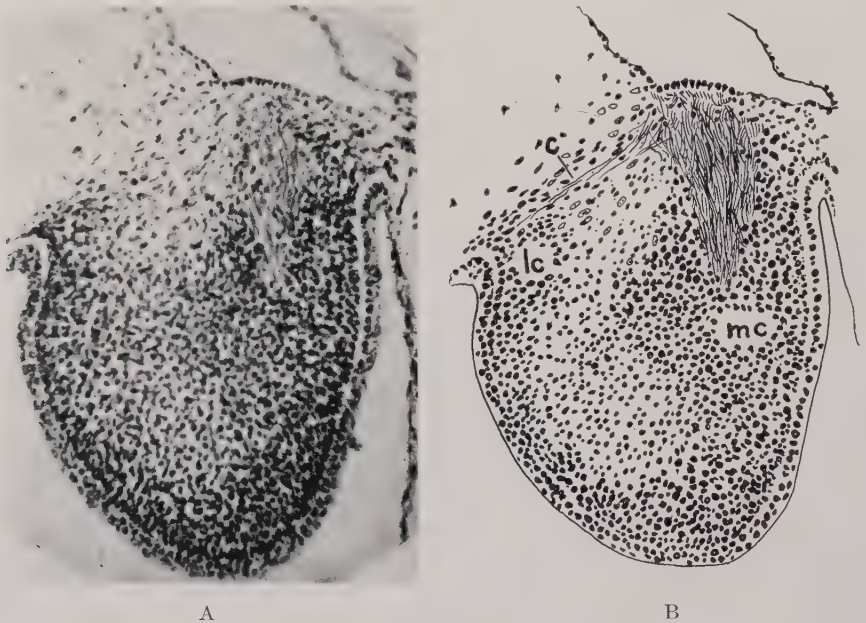


Fig. 4 Frontal section through the limb bud of a stage L 3 larva. A, photomicrograph to show the cellular details. Silver impregnation. $\times 150$. B, composite drawing to indicate extent of development of the innervation pattern. c, cruralis division. lc, lateral condensation of the mesenchyme. mc, median condensation of the mesenchyme.

having increased generally in thickness, especially at the lateral margin of the base and along the whole median surface of the bud. The thickening at the lateral margin extends medially and anteriorly into the bridge mesenchyme, its nuclei being greatly extended in that direction (lc, fig. 4). The median condensation extends from the tip to the base, with its greatest extent in the proximal half, where it reaches to the center of

the bud (mc, fig. 4). Into this more condensed mass of mesenchyme passes the main body of the nerve fibers from the plexus, the portion which represents the later sciatic division.

At this stage the fibers can be traced into the bud for about one-third of its length. Still very fine and taking only a slight impregnation, they are seen to intermingle within the plexus in a sort of feltwork. Yet, throughout the plexus and down into the limb bud, they are grouped into numerous fascicles, slightly separated from each other by open spaces. It is difficult to trace the individuality and continuity of the fascicles because of their number and irregularity and especially since fibers, singly or in small groups, frequently pass from one to the other.

Not all the fibers from the plexus enter the median mesoderm mass. A considerable number are turned laterally in the direction of the outer border of the limb bud, apparently following the stretched stroma in this region. This is the beginning of the cruralis division (c). Although it consists of numerous fibers where it leaves the plexus, this division tapers rapidly, so that only a very few of its fibers can be seen to have actually reached the condensed mesenchyme on the outer surface of the bud.

At stage L 3, no indications of a future innervation pattern are discernible other than this bifurcation of the nerve mass into the main cruralis and sciatic divisions. On the whole, the fibers form a fairly compact mass so that the advancing tip of the sciatic branch has roughly the shape of a cone. On the surface of the cone, and particularly at its tip, individual fibers are frequently seen leaving the mass to penetrate for a short distance between the cells of the mesenchyme.

At this point it should be mentioned that capillaries and small blood vessels are found in the limb bud as early as stage E 25, and are actively permeating the tissue throughout stages L 1 and L 2. By stage L 3, three main vessels, two venous and one arterial, have been established longitudinally through the limb with numerous smaller ones passing between. It is easily observed that the cruralis division of the nerves at this time

has no accompanying blood vessel, and the sciatic nerve, while it courses parallel to the sciatic vein through the same region of mesenchyme, is not applied closely to the vessel.

Stage L 4

While there is only slight change in external appearance of the limb bud during the interval between stages L 3 and L 4, several important developments are histologically apparent (fig. 5). The epidermis still shows a gradation from the embryonic condition at its tip to an almost fully developed larval condition near its base. At the lateral and dorsal regions of its base where differentiation has progressed farthest, the deeper layer has become pseudostratified, so that the nuclei are distributed in three layers.

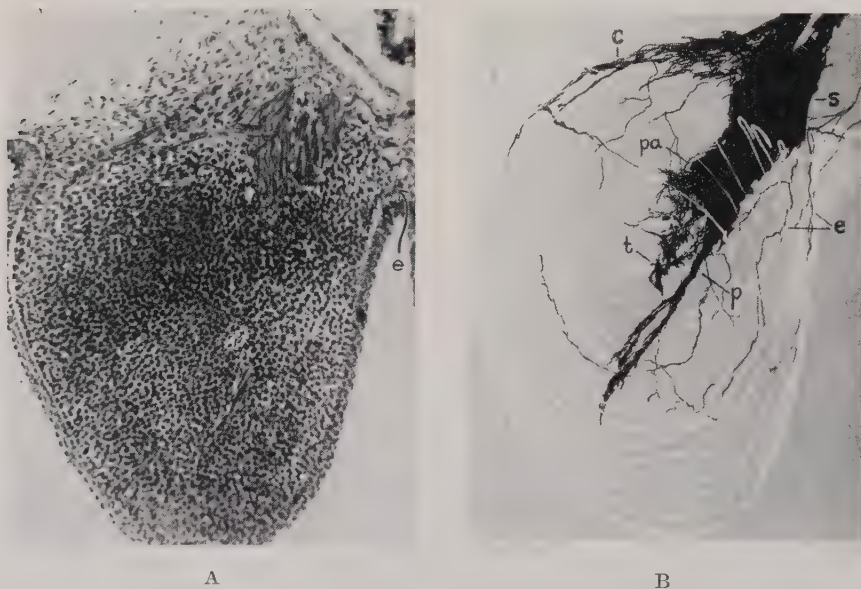


Fig. 5 Limb bud of a stage L 4 larva. A, photomicrograph of an 8-micra section impregnated with silver. $\times 110$. B, photograph of glass plate reconstruction to show extent of development of the innervation pattern. (White lines indicate the outlines of the limb bud). c, cruralis division. e, fibers of the epidermal primary innervation. p, peroneus brach. pa, profundus anterior branch. t, tibialis branch.

Distinct differences in the degree of compactness of the mesodermal nuclei make it possible by this time to identify the anlagen of the femur, and the tibia and fibula, and the larger muscle groups. Of these only the femur anlage gives any suggestion of its definitive shape, the others being merely centers of cellular condensation which can be identified only by their spatial relationships. These all appear to have differentiated from the mesodermal condensation, which in stage L 3 seemed to bud off from the median wall of the peripheral thickening and into which the sciatic nerve branch was then penetrating.

Along with these changes in the mesenchyme occurs a rapid development of the nerve pattern. Soon after stage L 3, the future nerve branches are foreshadowed by a splitting up of the peripheral portions of the nerve mass, accomplished apparently through a grouping of smaller fascicles already observed in the previous stage. A study of the condition of the pattern at stage L 4, in comparison with those of the following two stages, shows that what will be the main mixed trunks of the thigh and shank are already present, i.e., (1) the forking distal to the plexus into the cruralis and sciatic (c and s), (2) the division of the sciatic into the peroneus and tibialis (p and t), (3) the separation of the peroneus into the medial and lateral branches, and (4) the division of the tibialis into the profundus and superficialis (suralis) branches. Besides these, two purely cutaneous branches (Ramus cutaneous femoris lateralis and Ramus cutaneous lateralis)² can now be traced into tissue immediately subjacent to the epidermal layer.

One motor branch is clearly established. This appears as a tuft of fibers leaving the sciatic division near the point of its bifurcation into tibial and peroneal (pa). It penetrates for a short distance the denser mesenchyme lateral to the nerve mass and will eventually form the large profundus anterior branch to the extensor group of muscles in the thigh. No other purely motor branches are as yet distinguishable, unless one were to consider the sporadic single fibers which leave the

² Anatomical nomenclature according to Ecker und Wiedersheim (1896).

future mixed trunks irregularly along their courses to be the beginnings of muscle innervation.

Mention has been made of the appearance, at an earlier stage, of fibers within the epidermal layer. At this time, they are seen to enter the wall of the bud at its median border through two or three fine branches from a division of the eleventh spinal nerve. These branches (e), containing from one to three large fibers, course backward irregularly through the epidermis, giving off numerous branches. They do not reach to the lateral surface of the limb bud, but form a loose network in the epidermis of the medial surface and in the more rapidly proliferating tip epithelium.

In this stage, where the nerve branches are longer and more slender than in the preceding stage, it is even more evident that the courses of the newly forming nerve twigs and rapidly growing tips are independent of existing or developing blood vessels. For while the larger, more proximal branches of both systems tend to occupy the same tissue spaces, the finer and more distal branches, which represent the actively advancing portions, travel independently and may even cross at right angles.

Stage L 5

The mesodermal primordia in the limb bud of a stage L 5 larva have become much more distinctly outlined, so that it is possible to identify not only the skeletal elements of the thigh and shank, but also the muscle anlagen of the extensors and flexors of the hip and knee joints. Less distinct condensations at the distal end represent already the elements of the foot. Correlated with the condensation of mesenchyme in these primordia is the loose appearance of the tissue between them. It is within these spaces that the larger blood vessels and the nerves are now found to course.

The reconstruction of the nerve pattern of this stage (fig. 24) shows clearly that nearly all the branches of the adult leg have, by this time, been established. All of the cutaneous branches can be identified, and all of them traced into the

dermis. Three other branches (shown to be sensory by experiments to be published elsewhere), which in older stages can be traced into the ligaments and tendons of the knee and ankle joints, are also well established. This is rather surprising since, even in the adult frog, these branches are so small that only one of them has so far been found described in the literature. This latter is the *Ramus articularis genu et pedis*, mentioned by Ecker (1896). It leaves the peroneus near the knee joint. The other two of these, presumably proprioceptive nerves, run to the knee, one from the peroneus, which will supply the antero-lateral surface of the joint, and the other from the *tibialis profundus* to its median region. These might be designated the *Rami articulares genu lateralis* and *medialis*, respectively.

Most of the motor branches are also represented. The larger ones are short projections from the mixed trunks, which taper rapidly to a point as they penetrate into the muscle anlagen, while the smaller ones consist of a few frayed-out fibers, which leave the mixed branches along their courses. These branches are more difficult to identify because of their relative shortness and the fact that the muscles which they supply are not clearly segregated. On the whole, the motor branches seem less well established than those to the skin.

The few epidermal fibers derived from the eleventh spinal nerve are distributed along the medial surface of the limb bud (e, fig. 24). None of them extend to the skin of the lateral surface.

Stage L 6

In the tadpole of stage L 6, when externally there appear first indications of a foot paddle, all motor and sensory branches are distinctly identifiable (fig. 25). Furthermore, the increase in the limb bud length, which has occurred since stage L 5, has resulted in the beginning of a process of drawing out of the nerves to assume more nearly their final proportions.

Most of the cutaneous branches have not only reached the skin, but have coursed along its inner surface, giving off collateral branches at intervals. The extent of the epidermal nerves from the eleventh spinal segment is very similar to that in stage L 5. These fibers persist at least until late larval life, becoming progressively less conspicuous. By the time the toes are fully formed, they consist of no more than two or three single fibers on the median surface of the leg, branching only in the foot and toes. Apparently they are wholly independent of the leg innervation supplied through the sciatic plexus. There appears to be no functional significance attached to this dual source of innervation since tests on tadpoles of various ages showed no difference in response to stimulation of the medial and lateral surfaces. Metamorphosed frogs have not been examined to determine whether this innervation is retained in the adult.

Along with the lengthening of the leg, its muscle primordia are also extended longitudinally. Although origins and insertions have not yet differentiated, the myoblasts of the future muscle belly have assumed spindle shape and, particularly in the region of the thigh, cross striations are beginning to appear. Correlated with these muscle developments, there has been a lengthening of the nerve branches innervating them. The forking of the larger motor branches, which in stage L 5 had barely begun, is here well established. Furthermore, the tips of many motor fibers are associated with the newly striated muscle fibers in a fashion quite typical of mature amphibian motor endings. It might be pointed out here that no movement of the limb occurs until three stages later (stage L 9).

As far as concerns the innervation of the limb, with the possible exception of the foot, the pattern foundation is essentially complete by stage L 6. It is, therefore, unnecessary to describe here the later stages, in which the systems already laid down are merely filled out and extended.

THE SOURCE OF LIMB INNERVATION

We are not here concerned with the time or manner in which the spinal nerves first make their appearance, since this occurs comparatively early in embryonic life, long before the limb primordia appear. What does interest us is the manner in which the limb bud, situated as it is on the ventral surface and developing at a relatively late larval period, will be furnished with innervation from neurones located in the spinal cord and ganglia of the lumbar region. At an early stage the direction and course of spinal nerves 8, 9, and 10, which are destined to innervate the leg, are not, in general, different from nerves of most other segments. That is to say, they run posteriorly and ventrally between the notochord and myotomes, then laterally between the somatopleure and body wall mesenchyme. The convergence of the eighth, ninth, and tenth nerves to the site of the future limb would appear to be a natural result of the architecture of that region, since the coelomic linings of the right and left body walls themselves converge and unite at the region of the cloaca. Such an interpretation would be in line with the emphasis on the role played by the mechanical configuration of embryonic organs and tissues in the orientation of nerve outgrowth found in the writings of His (1887) and Harrison ('10 and '14) and more recently elaborated by Weiss ('34, '41). In this way the appropriate spinal nerves are already brought into the vicinity of the future limb bud before the appearance of that organ. The active proliferation within the tissue of the limb bud anlage, after it first appears in stages E 25 and L 1, is then able, by means of chemical and mechanical actions to effect micellar orientation within the surrounding stroma in such a way as to act as a passive local collector of all growing fiber tips within its reach (Weiss, '34).

Reference again to the figures of early stages will show that the fibrillae of the nerve mass growing into the mesenchyme bridge have an orientation that coincides with the arrangement of the background of cell strands; that the advancing fiber tips follow the configuration of the mesostroma can clearly

be seen in the illustration of stage L 3 (fig. 4), particularly in the region of the newly appearing cruralis division.

It has already been stated that limb buds of anurans appear relatively much later in ontogeny than in other amphibians. In the frog embryo, a primary innervation for the trunk musculature and skin is established and is functional long before the appearance of hind limb primordia. It is the fibers of this primary somatic innervation, extending from the cord to the region surrounding the future limb, which then serve as a conducting pathway for the later fiber outgrowth in a manner which Weiss ('41) has called "growth by application". The latter secondary innervation becomes visible in silver preparations soon after the appearance of limb primordia and is destined to supply the limbs.

The fibers thus furnished to the early limb bud consist of axons of both sensory and motor neurones. This was determined by tracing the fibers back to both the dorsal and ventral roots, as described below, and by histological studies of tadpoles in which the fibers coming from the cord had been allowed to degenerate after unilateral section of the ventral roots supplying the sciatic plexus. Such animals showed that the volume of fibers at the level of the limb bud was smaller on the operated side than on the control side, a condition which would be true only if motor as well as sensory fibers had originally been present.

It has been noted that all the fibers entering the limb bud through the sciatic plexus are very fine and take a light silver impregnation. The contrast in size between these and the fibers which supply the adjacent body wall and musculature is very marked when they appear side by side in the mixed nerves above the plexus. A cross section of the ninth spinal nerve of a larva in stage L 2 is shown in figure 9, from which it is seen that the fiber diameters may differ by several hundred per cent. By means of this difference, it is possible to trace the limb bud innervation back to its sources in the ganglia and cord. With growth of the larva, there is progressive increase in diameter of fine fibers. The most rapid change occurs

between stages L 1 and L 4, followed by a comparatively gradual increase between L 4 and L 16. On the other hand, there is a more steady augmentation of the number of these fibers up to at least stage L 16 (fig. 13).

These changes in size and number of spinal nerve fibers are intimately related to changes taking place in the ganglia. At the beginning of stage L 1, the spinal ganglia of nerves 8, 9, and 10 contain comparatively few cells. About twenty-five to thirty of these are large and already possess large nuclei and distinctly visible processes. They make up the bulk of the ganglion; the remaining cells are smaller and stain more darkly in azan. By stage L 3 there has been a considerable increase in the number of the smaller cells while the larger ones, which are isolated in a lateral cluster, still number approximately thirty or less. The increase of the small cells continues steadily. By actual count, in the ninth ganglion of a stage L 6 tadpole, there were twenty-three large and 2,190 small cells (fig. 10). That many of the small cells are real ganglion cells is already evident before stage L 6 since fibers can, by this time, be seen to originate from them. Clearly these are the cell bodies of some of the fine fibers found in the spinal nerve, while the heavy fibers are processes of the large ganglion cells. A similar set of large and small fibers extend from the ganglion up the dorsal root to the sensory column of the cord. The number of large fibers in this root is the same as the number of large cells in the ganglion and remains fairly constant in all early larval stages, while the number of small fibers increases with the number of small cells in the ganglion.

The ventral roots are likewise composed of two sizes of fibers comparable to those in the dorsal roots. The large fibers come from the region of the motor neurones lying in the ventral gray matter and leave the cord just lateral to the median ventral fissure. The fine fibers leave the cord by several small branches at points more dorsal and lateral. They unite and join the bundle of larger fibers in their course toward the ganglion. The number of heavy fibers in a mixed nerve trunk is equal to the sum of those in its dorsal and ventral roots.

Figure 14 shows a diagrammatic representation of the relationship of these fibers to their cell bodies and end organs.

The description of spinal nerves and their ganglia given above holds only for the lumbar and brachial regions. Cross sections of spinal nerves 5, 6, 7, and 12 differ markedly from those of the limb segments in the comparative scarcity of fine fibers (figs. 7-9). The ganglia of these segments contain correspondingly few small cells (fig. 6).

In studying the ventral roots of the spinal nerves of young amblystoma larvae, Youngstrom ('40) finds a new system of fine motor fibers appearing among the older fibers of the primary innervation of the embryo. Because this secondary innervation appears at the time of the development of independent withdrawal reflexes in the limb, he considers it the mechanism for the individuation of localized movements from an earlier more generalized behavior pattern. In the larva of *Rana pipiens*, however, where the nerves have been followed to their peripheral endings, heavy fibers can be traced to only those end-organs which are functionally innervated during the embryonic period, namely, somites and skin, while the newly developing larval limbs are exclusively supplied by the

Figs. 6-13 Spinal ganglia and nerves from larvae of various stages to show the size difference between the cell bodies and fibers of the primary and secondary innervation. Silver impregnation according to Bodian.

Fig. 6 Sixth ganglion of a stage L 2 larva. (Does not furnish fibers to limbs.) Note relatively small number of small secondary cells. $\times 340$.

Fig. 7 Cross section of spinal nerve of same segment showing very few fine fibers. $\times 340$.

Fig. 8 Ninth ganglion of stage L 12 larva. (Furnishes fibers to hind limb.) Note comparatively large number of small secondary cells. $\times 340$.

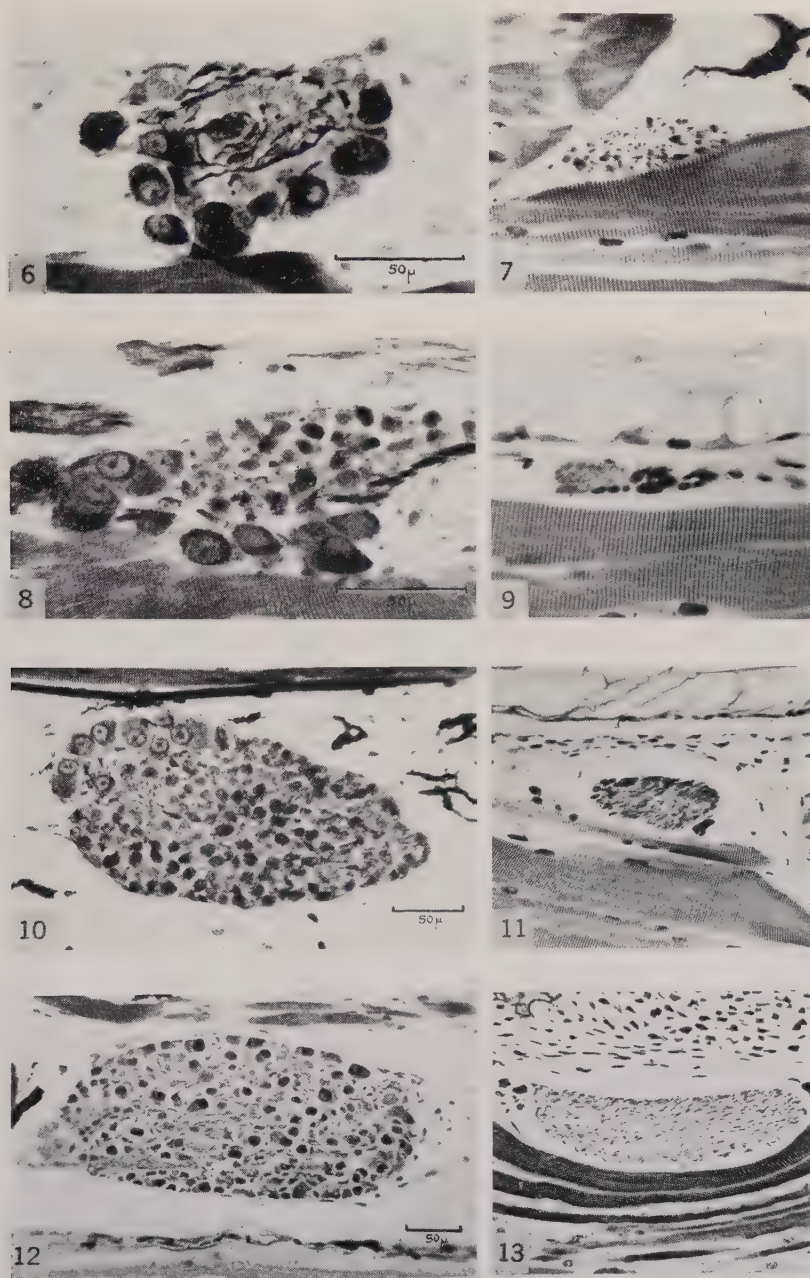
Fig. 9 Cross section of spinal nerve of same segment. Note the mass of very fine fibers beside the few large ones. $\times 340$.

Fig. 10 Ninth ganglion of a stage L 6 larva. Contrast the number of secondary ganglion cells with the cluster of comparatively large primary cells at the left. $\times 190$.

Fig. 11 Cross section of spinal nerve from same segment. Note the increase in size of the secondary fibers over those of stage L 2. $\times 190$.

Fig. 12 Ninth ganglion of a stage L 16 larva. Primary cells not easily distinguishable from the secondary. $\times 150$.

Fig. 13 Cross section of spinal nerve of same segment. Note small number of large primary fibers still present. (At left.) $\times 150$.



Figures 6 to 13

fine fibers of the secondary innervation. Since in the frog limb there are no primary motor fibers prior to the establishment of the definitive innervation, there seems to be no anatomical evidence for the assumption that localized behavior patterns of the limb are preceded by a generalized total pattern of true limb movements mediated through a special nervous mechanism.

An attempt to correlate the development changes in the limb bud with those taking place in their source of innervation leads to the following conclusions. Prior to the appearance of the hind limb primordium, a neural pathway has been in existence, extending from the source of fibers to the very

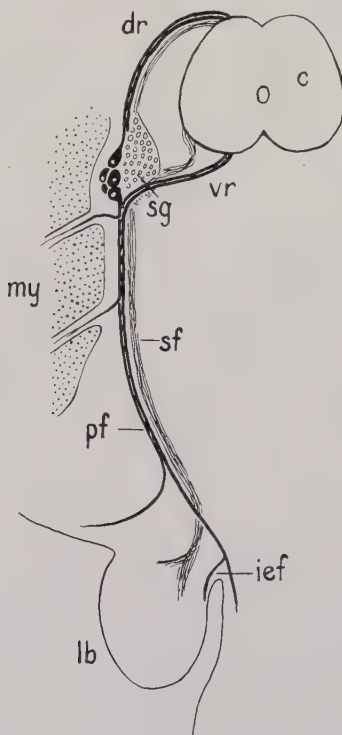


Fig. 14 Schematic drawing to illustrate the relationship of the primary and secondary fibers to the cord, ganglia and end-organs. c, spinal cord. dr, dorsal root. ief, intra-epidermal primary fibers. lb, limb bud. my, myotomes. pf, large primary fibers. sf, fine secondary fibers. sg, spinal ganglion. vr, ventral root.

“door-step” of the future limb. The nerve fibers which form this “pathway” are part of a primary embryonic innervation which has established early end connections with adjacent trunk muscles and skin, but will never enter the leg. At about the same time that limb bud anlagen are to appear, a mass of new cells begin to differentiate in the ganglion, and their axons, along with similar fine fibers originating from the cord, following the “pathway” by application, are conducted toward the base of the new limb.

NERVE PATTERN FORMATION

The definitive nerve pattern of the frog limb consists of many divisions, trunks, and branches. In its development, each of these is established at a different time in different parts of the limb bud, and hence, under varying local conditions. Each part encounters different obstacles and is subjected to differential stretches and shifts as the elements of the limb increase in size and alter their relative positions. The process of pattern formation consequently must be approached through a consideration of the factors operating in the establishment of each of its branches separately. In analyzing the mechanics of nerve growth and pattern formation, Weiss ('41) summarizes the process of establishment of an embryonic nerve in the following words:

“Thus the establishment of a peripheral nerve involves three overlapping phases: First, the free outgrowth of a group of pioneering fibers through non-nervous surroundings; second, the bound outgrowth of subsequent fiber generations along the line laid down by the pioneering fibers; and third, the towing process in which the nerve is drawn out by the growth and dislocations of its terminal tissues.”

If the forces determining the end attachment of nerves act only on their pioneer fibers, the agencies responsible for the basic configuration of nerve pattern in the limb must be active at a time preceding the building up, or filling out of its branches. From the description of limb development here presented, this would necessarily be at a very early stage.

Is it quite obvious, then, that pattern development cannot be thought of as an invasion by nerves of a visibly differentiated limb, in which the configuration of the nerves is determined by the spatial arrangement of limb elements already present, e.g., blood vessels, muscles or skin. Such a conception seems to be held by some who, like Hamburger ('28), have drawn inferences mainly from terminal stages. One should, rather, think of the visible differentiation of limb elements as occurring within mesenchyme which has previously been invaded by pioneer nerve fibers and in which nerve branches are already forming.

It remains then to discover the forces which may operate to establish the courses of the pioneer fibers, and the factors which will produce from these the nerves of the final pattern.

Various agencies have been proposed by different authors to account for the apparently directed outgrowth of nerves. Most prominent among these are the theories of Galvanotropism (Kappers, '17; Child, '21), Chemotropism (Cajal, 1893, '08; Tello, '23) and contact guidance (His, 1887; Harrison, '10, '14; Weiss, '33, '34). Reviews evaluating the different theories in the light of more recent experimental work are presented by Harrison ('35), Detwiler ('36), and Weiss ('41). They agree that all the direct experimental evidence at hand upholds a contact theory of orientation (Young, '42). Studies on the behavior of living nerve fibers carried out by Weiss in tissue culture ('34) and in living animals ('41) have revealed some of these factors which are capable of directing the free outgrowth of the pioneering fibers. In general, they consist of interfaces in the environing tissue which may serve as solid substrata for the amoeboid progression of the fiber tips. Such interfacial orientation may be brought about by a variety of vectorial forces, such as mechanical stretch, dehydration and other colloid chemical changes.

It was indicated in the description of stage L 1 tadpoles that even before the visible ends of the ingrowing nerve mass enter the mesenchyme of the limb bud, there exists a very definite structural organization of the mesostroma, probably

related to the course of migration of the mesenchyme cells. During the interval between stage L 1 and L 3, shifts of the limb bud result in mechanical stresses which intensify and alter the configuration of the ground structure, particularly in the region of the mesenchyme bridge. That these definitely oriented finer structural elements are related to the subsequent course of nerve fibers which soon enter and pass through the bridge, is suggested by the congruity of their patterns. The alignment of the stroma strands is much more clear cut along the medial and lateral borders of the bridge than in its interior. The appearance within these regions of the two main divisions which emerge from the sciatic plexus is a notable instance of a definite relationship between the pattern of the preneuronal substratum and the developing pattern of the ingrowing nerve fibers.

Although less strikingly apparent and not yet adequately studied, local variations can be seen in the arrangement of mesostroma within the limb bud proper, which may be, in part, responsible for the course of the nerve branches to develop later in the region peripheral to the bridge.

Because the fate of the future nerve branches and, hence, of the whole pattern, rests in large part upon the course taken, and the end attachment made, by the tips of the pioneering fibers, the importance of a clear understanding of their characteristics and behavior is evident. Yet definite knowledge concerning the character of fiber terminations is very meager, due largely to the inability to trace them under the microscope to their actual endings.

Boeke ('30, '37), Stöhr ('35) and Nonidez ('37) have studied the terminations of sympathetic and somatic fibers in fixed and stained preparations. Among these workers there is disagreement concerning the structure of nerve fiber endings. The complete individuality of each fiber implied in the neurone theory is questioned by Boeke and Stöhr, who describe terminal anastomosing in the peripheral sympathetic plexus. Boeke ('37) has also found in the early phase of peripheral nerve regeneration an apparent syncytial anastomosing of the

fibers at their terminations. Later, from this primordial plexus, the definite nerve fibers are individualized.

The possibility that such a condition may also exist in embryonic organs at a time when they are first invaded by nerve outgrowths must be conceded. Certain observations on the silver impregnations of early tadpole limbs would at least bear such an interpretation. In stages L 2, L 3, and L 4 faint depositions of silver are visible along extremely fine strands of a fibrous nature, which interlace and anastomose between the mesenchyme cells and which appear to be continuous with the early nerve fibers. Connective tissue within the limb primordium has not yet differentiated its fibers, and the mucoid strands, which are present, have a different appearance from these argentophil fibrillae. There is thus a suggestion that at a very early period in the innervation of the limb, the invading nerves form a reticulum of exceedingly fine neuroplasmic strands within the undifferentiated mesenchyme from which may be individualized the larger discrete fibers following the chemical differentiation of the mesenchyme.

The dearth of knowledge concerning the character of nerve fiber terminations makes it impossible to form a clear picture of the manner or time of their connection with cells of future end-organs. It does seem certain, however, that the actual contact between a fiber and its embryonic end-organ cells is accomplished before there is microscopically visible evidence of this connection.

Although we are unable to determine the exact moment when a pioneer fiber is first connected with its end-organ cells, certain changes soon follow which indicate that such a connection has already been made. It has been suggested by Weiss ('41) that the establishment of its end connection changes the character of a fiber in such a way as to induce other similar fibers to grow out along its surface by "selective fasciculation". The importance of this process in the building up and filling out of the nerve branch has already been emphasized. Thus, the appearance of a fiber cable in the place of independent fibers

would indicate that terminal attachment of the pioneer fiber has been made.

It is possible to tell approximately the stage in larval limb development when the major branches have become certainly established. By stage L 5, every branch of the thigh and shank is present. This may also be true of the foot. In stage L 4, the main divisions and many of their branches are distinctly visible, while probably some of the smaller collateral branches are not yet established. At stage L 3, only the representatives of the large cruralis and sciatic trunks are visible. There are no divisions of the sciatic trunk. From the cruralis trunk, however, a few fibers can be seen to leave at the relative position of the future muscle branch. It is apparent in this case that the cutaneous branches of the trunk precede the muscle branches in their establishment. The relative size of the two types of branches in later stages would agree with this. In stage L 2, no signs of any division of the nerve mass can be seen.

The conditions found at these graded stages would indicate a progressive establishment of nerve branches, beginning at the basal portion in stage L 3 and proceeding distally to completion of the pattern in stage L 5. If then the appearance of these branches indicates the previous anchoring of their pioneer fibers, this process must have occurred prior to stage L 3 for at least the two major divisions. This would be long before there is any visible differentiation of the mesenchyme.

The divisions and branches as established at this early period do not resemble, in their spatial relationships, the mature innervation pattern of the limb. The process by which this preformed nerve pattern is unfolded, or, more correctly, drawn out, can only be understood in terms of the extensive changes in size and shifts in relative position of the muscle and skeletal elements accompanying the growth of the leg. When first formed, the trunks and divisions are short and their branches leave them near their bases. Such a condition results from the fact that the primordia of the future limb elements are arranged side by side in the shallow limb bud. This

telescoped condition of the nerve pattern is immediately apparent from examination of the reconstruction of as late a stage as L 5 although it is more striking in stage L 4. Even in stage L 6, it is seen that the sciatic plexus is close to the limb bud base and that the cruralis division leaves the sciatic at this level. Recalling the condition in the metamorphosed frog, it is apparent that the relative distance between the plexus and the limb has been greatly increased, and that the cruralis has been routed a considerable distance around, dorsal to the iliac bone, before entering the limb proper. In fact, the precartilage of the acetabulum forms within the limb bud (stages L 5 and L 6) and then is left behind by the migration of the whole limb.

Through the lengthening of the leg by muscle shifts, differential growth and the consequent serial alignment of its component elements, the nerve branches anchored to them are towed out to form the main longitudinal trunks with the branches distributed along their length. The effect of this stretching out of the nerve pattern during growth of the limb is diagrammed in figure 15. In this process certain distortions may occur. If the basal portion of a branch comes to lie parallel to the mixed nerve from which it forks, it may later be bound together with the larger nerve by connective tissue wrappings, and thus its point of divergence is shifted distally. On the other hand, an obstacle becoming caught in the fork may separate the fibers of the two branches, causing them to diverge at a relatively more proximal point.

It is obvious that the factors involved in this last phase of pattern formation are those of the mechanics of differential growth of the non nervous elements. As such they have little to do with the determination of the original basic pattern, their effects being expressed only in the arrangement of the nerve bundles trailing behind the growing muscles and skin, and in packing them, together with the blood vessels, into the spaces between the other limb elements.

Our observations of the mutual independence of the courses of blood vessels and nerves during the critical stages in which the nerve pattern is laid down, seem to deprive all claims that

blood vessels are primary agents of nerve patterns of their factual foundations.

This paper, being primarily descriptive, has touched on only a few of many unsolved problems relative to the development of the innervation pattern. Some of these will be discussed in the experimental part of this work to be published later.

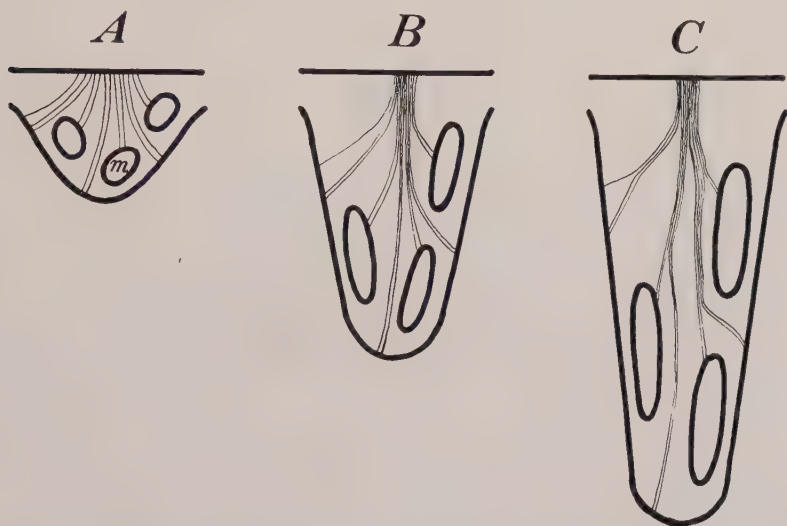


Fig. 15 Diagram to illustrate the effect upon the innervation pattern of the growth and accompanying positional shifts of limb parts. A, nerve pattern when first established at a very early stage. B, intermediate stage. C, late stage with the nerve trunks, divisions, and branches in their definitive positions. m, muscle anlage.

SUMMARY

Since no detailed study of the development of limb nerves in the frog larva has previously been made, it is here undertaken as a preliminary investigation for experimental work to be reported elsewhere.

A study of serially sectioned limb buds of larvae at different stages showed that the establishment of nerve pattern occurs very early, commencing before stage L 3, when as yet there is no tissue differentiation in the mesenchyme of the limb bud, and being essentially completed by stage L 5.

From a consideration of the process of nerve outgrowth in the light of recent experimental work, it is concluded that:

1. The determination of the various branches in the innervation pattern of the limb occurs prior to the time of visible appearance of the branches.

2. The factors which direct the outgrowth of the early fibers and determine the branches of the nerve pattern are to be found in the preexisting pattern in the mesostroma.

3. The possibility of the presence of a neuroplasmic reticulum within the early limb bud mesenchyme, giving rise later to the individualized nerve fibers, is suggested.

4. The determination and establishment of the innervation pattern is not simultaneous for the whole system, but progresses, in general, from the base to the tip.

5. The pattern thus determined is then altered and shaped by the mechanical forces accompanying the differential growth of the parts of the limb.

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PLATE 1

EXPLANATION OF FIGURES

16-23 Unretouched photographs of the tadpole limb at different stages of development. $\times 22$.

16 Stage L 2. The length of limb bud is equal to one-half its diameter.

17 Stage L 3. The length of limb bud is equal to its diameter.

18 Stage L 4. The length of the limb bud is one and one-half times its diameter.

19 Stage L 5. The length of the limb bud is equal to two times its diameter. A slight ventral bend of the distal half of the bud appears. There is no foot paddle flattening.

20 Stage L 6. A flattening of the distal end of the bud is noticeable. No interdigital notches yet present.

21 Stage L 7. Slight indentation between the fourth and fifth toe prominence (at point indicated by arrow).

22 Stage L 9. Interdigital notches 2-3, 3-4, and 4-5 present.

23 Stage L 12.

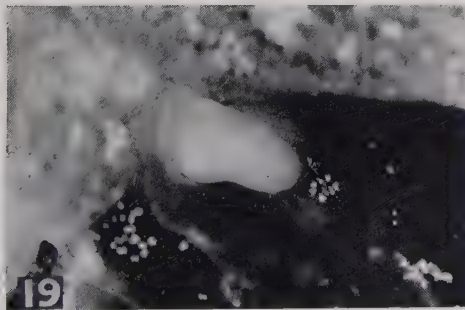
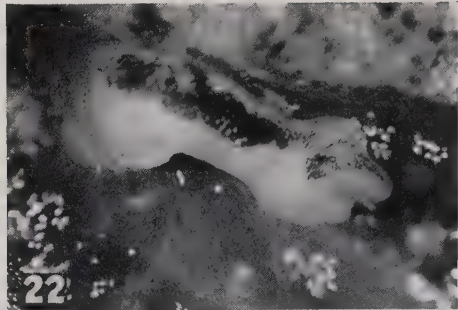
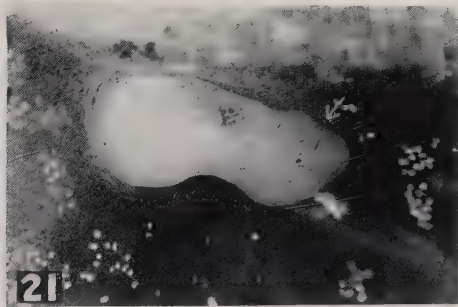
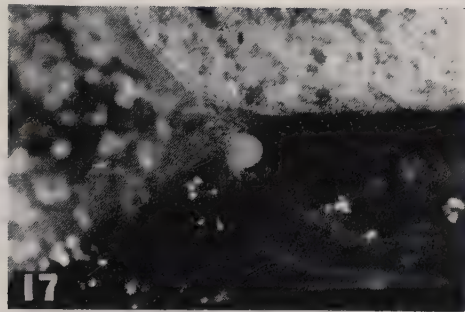
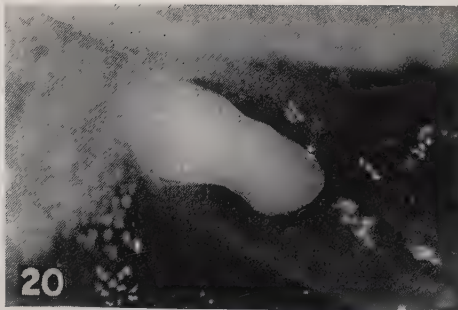
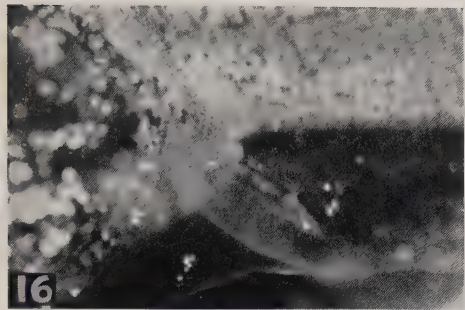
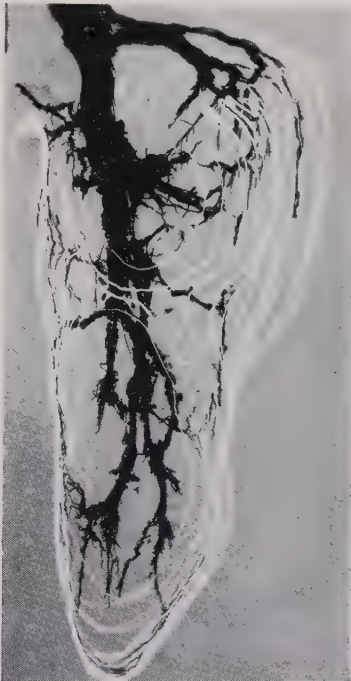


PLATE 2

EXPLANATION OF FIGURES

- 24 Stereoscopic photographs of glass plate reconstruction of a stage L 5 limb bud. (White lines represent limb bud outlines.) e, intra-epidermal primary fibers.
- 25 Stereoscopic photographs of glass plate reconstruction of a stage L 6 limb bud.



GONAD AND RELATED ENDOCRINE RESPONSE OF FEMALE RATS TO EXPERIMENTAL HYPERADRENALISM

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TWO PLATES (TWELVE FIGURES)

The antagonistic action of adrenalin on the reproductive system of female mice has been reported by Robson ('32). He found a suppression of the estrous cycle and an accompanying regression of ovarian activity. The writer ('41a) found that injections of adrenalin into both sexes of sparrows at the height of gonadal development were followed by a return to a condition comparable to the quiescent winter state. The writer ('41b) reported definite although variable gonadal regression in male rats following treatment with adrenalin. Quite generally the accessory reproductive organs became atrophic, and in many instances testicular atrophy similar to a cryptorchid response was apparent. Wheeler, Searcy, and Andrews ('42) have studied the effects of adrenalin upon semen production in the fowl. They report a 48% decrease of semen volume at the end of a 30-day injection period. They also noted a marked reduction in concentration, number, and survival time of the spermatozoa. Dissociation of the cells of the seminiferous tubules up to 7 weeks after the cessation of injections was observed. The present report is concerned with the effects of experimental hyperadrenalism on the female rat reproductive system.

EXPERIMENTAL PROCEDURE

Daily injections of Parke, Davis Adrenalin Chloride (1:1000)¹ were given to twenty-two adult female rats from five selected litters. Initially the animals were given a 1 minim injection to accustom them to the hormone. After 3 days the dosage was increased to 2 minims and maintained as such on all successive days for all animals. It had been found (Perry, '41b) that optimal results in male rats were obtained with daily 2 minim doses. Daily injections of 4 minims were not as effective in male rats as were those of 2 minims. Hence the variations in response of the different animals of this report is not due to differences in dosage. A control animal from each litter was not injected. Daily vaginal smears were recorded. The animals were sacrificed when the vaginal response indicated inhibition of gonadal activity was operative. At autopsy the reproductive tracts, pituitaries, thyroids, pancreas and adrenals were fixed in suitable fixatives. The weights of organs were recorded from 70% alcohol. Stains employed were Ehrlich's hematoxylin and eosin for routine study; Gomori's pancreatic stain ('39) for pancreatic islands, pituitaries, adrenals, and thyroids; Crossmon's stain ('39) was also used for pituitaries. Tests on the metabolic rate of experimental and control animals were carried out according to the Haldane method. Blood sugar determinations were made according to the La Motte method. Body weights were recorded at the start of the experiments, periodically during the course of experimentation, and at the time of sacrifice. Particular attention was given to sanitary and dietary requirements. The special rat diet produced by the Maritime Milling Co., Buffalo, New York, was used for these and all other rats of our colony.

RESULTS

From the vaginal smear record a close tabulation of ovarian activity could be kept. Universally, such tabulation showed an interruption of the estrous cycles. They became irregular

¹The writer is indebted to Dr. Oliver Kamm; Parke, Davis and Company, Detroit, Mich., for a generous supply of Adrenalin Chloride.

and were characterized by long periods of diestrus. Interestingly enough the rats of each litter responded in a similar manner. It seems a usual occurrence of normal rats to show similar smear reactions when caged together. The animals of litter 1 exhibited regularly recurring periods of diestrus of 5–8 days duration, interrupted by partial or complete estrous cycles. Litters 2 and 3 behaved in like manner except that the diestrus periods were longer, 12–14 days. Litter 4 showed a diestrus range of 19 to 29 days. Litter 5 was characterized by a continuous diestrus period beginning shortly after the injections and lasting until the time of sacrifice, 30 to 40 days later. A composite idea of the efficacy of adrenalin inhibition may be gained by considering the ratio of the total number of days the animal is in diestrus—during the injection period—to the number of days the animal is in proestrus, estrus, and postestrus combined. For convenience this ratio may be termed the “diestrus ratio”. Our control animals exhibited a 1:3 diestrus ratio. These ratios for the treated animals were: litters 1, 2, and 3, 1:1; litter 4, 2:1; litter 5, 12:1.

The health of the experimental animals was not impaired in any detectable manner. There were no signs of vitamin deficiency. There was no evident loss of appetite. There were no skin sores at the sites of injections or elsewhere. It is true that the experimental animals did not keep pace with the control animals in all instances. In view of the fact that the metabolic rate of injected animals, as reported below, was higher than that of controls it would seem that the percentage weight differences of experimental animals as shown in table 1 is not excessive.

The surprisingly long diestrus periods in litter 5 require explanation. At the present time there are no clear-cut experimental facts known to the writer which might clarify these long periods. However, certain possible solutions are discussed below. It may be noted that a comparable proportion of male rats studied in previous investigations exhibited severe testicular damage in response to adrenalin. A majority

the quantitative magnitude of the adrenalin antagonism in other animals.

Inspection of the last two columns reveals that adrenalin inhibitions are operative in animals of all litters. These quantitative results, especially when considered in relation to qualitative differences, seem to indicate that atrophic changes in the reproductive tract are characteristic responses of hyperadrenalism. Consideration of the photographic results will reveal the extent of these qualitative changes.

Ovarian effects. The ovaries of treated animals exhibit follicular atrophy. The degree of atrophy corresponds in general to the loss of ovarian weight, and it also seems to bear a relation to the length and number of diestrus periods. Follicular regression is evident in the experimental ovary when compared to the control ovary (cf. figs. 3 and 4). The interstitial cells of the ovaries of experimental animals from all litters exhibit the "deficiency reaction" (cf. figs. 5 and 6). This reaction is characterized by the "wheel-like" and beaded appearance of the nuclei of interstitial cells. According to Collip et al. ('38) the presence of such nuclei is a sign of failure of pituitary gonadotrophic function. Consideration of figures 7 and 8 indicate that the corpora of adrenalin injected rats also exhibit a reaction that may be termed a "deficiency reaction". Instead of the typical luteal cells the corpora lutea of injected animals show cells with pyknotic nuclei and considerable numbers of fibroblast-like cells. Robson ('32) also observed this reaction in mouse ovaries of adrenalin treated animals. The above reactions point to a suppression of both pituitary gonadotrophins — F. S. H. and L. H.

Accessory effects. A comparison of figures 9 and 10 will emphasize the changes in the uterus, not only of gross size but also of histological structure. In the experimental animal the uterus has regressed to the size of a 5- or 6-week-old immature animal. There is a marked decrease in the density and number of cells. The layers of the myometrium are clear and distinct; the endometrium has taken on a fibrous character.

The vagina of the injected animals (fig. 12) has become smaller in diameter, and its lumen is correspondingly narrower. A peculiar and seeming fusion of the vaginal epithelium, as evidenced by the closure of the crypt-like structures in the walls, might indicate an attempt to close the lumen. A similar condition is encountered in the vas deferens of maximally responding adrenalin injected males.

ADDITIONAL ENDOCRINE EFFECTS

The pituitaries of the litter exhibiting maximal response to the injections decreased greatly in weight as compared to the control animal. The pituitary of the control weighed 7 mg. while the pituitaries of treated litter-mates ranged from 2 to 3 mg. A paucity of basophiles in these latter was evident. Pituitaries of other injected animals were consistently lighter than those of their respective controls, however, the basophiles were not markedly reduced in number. Similar results have been found in the pituitaries of adrenalin injected males.

The thyroids in maximally responding animals were hyperplastic. The most significant thyroid effect was apparently a prolonged elevation of the metabolic rate. Determinations of metabolic rate were commonly made on a sixth litter. Before making such determinations all food and water was withdrawn for 18 hours. Elevation of the metabolic rate followed individual injections. After the cessation of the injections the metabolic rate remained above normal for a period of 20 days before returning to normal. These results were judged by the cubic centimeters of oxygen consumed per gram of body weight per hour. The peak consumption occurred around the fourth or fifth day after the cessation of injections. Seemingly the adrenalin had stimulated the thyroid to an increased production of its hormone which, once it was attained, persisted for 3 weeks. A similar prolonged elevation of the blood sugar was not observed. An elevation of blood sugar after single injections attained a peak between 1 and 2 hours after treatment. A return to normal occurred 6 hours after injection. An indication of an increase was observed in the insulin pro-

ducing cells of the pancreatic islands. These cells were more numerous in the islands of several treated animals than they were in the islands of control animals. An insufficient number of animals has been studied to conclude that such an increase of cells is typical. However it seems logical to suppose that this would be a likely response.

DISCUSSION

From the foregoing results it seems that adrenalin exerts an antagonistic action on the female rat reproductive system. The resultant inhibition seems to be more evident in the production of morphological changes than in the suppression of function. This is indicated by a generally uniform atrophy of ovarian and accessory structures, whereas the suppression of estrus is not continuous in all animals. It has been noted (Perry, '41a) that the degree of regression in sparrows is approximately the same in all animals. An explanation of the unequal reactions in rats, both male and female, is desirable. This variability may be due to genetic factors, or it may be due to certain endocrine interrelationships. If genetic factors are involved it may be that some animals inherit a sensitivity or susceptibility to adrenalin and other gonadal inhibitors. Concerning possible endocrine interrelationships, it was noted that animals with a high (12:1) diestrus ratio had very light pituitary weights (2 to 3 mg.) and very few pituitary basophiles. The results generally indicate this gland to be the site of the adrenalin antagonism as evidenced in the follicular atrophy and the "deficiency reactions" of the interstitial cells and the corpora. Robson ('32) concurs in this view. It would seem therefore that the explanation of the variations observed is to be sought in the degree of pituitary inhibition caused by adrenalin alone, or in conjunction with other antagonists. Smelser ('39) has presented evidence that hyperthyroidism in male rats is followed by gonadal inhibition presumably of pituitary origin. Hyperplastic thyroids were found in rats having a 12:1 diestrus ratio. This may be of significance in explaining the maximal response of these rats.

It would seem to indicate some sort of synergism between thyroid and adrenal glands at least with regard to pituitary inhibition. Goldzieher ('39) calls attention to several instances of a synergism between the thyroid and adrenal medulla. In his book "The Endocrine Glands" he states

page 622; "Definite synergism seems to exist between the adrenal medulla and the thyroid. There is no adequate response to adrenalin in the completely thyroidectomized animal (Eppinger and Linne). On the other hand, thyroxin, if given over a period of time, increases both the pressor and glycemic response to adrenalin (Kraus and Friedenthal). This is in accord with the frequency of combined hypertension and hyperglycemia in cases of chronic hyperthyroidism."

Recent experiments of the writer have further indicated that feeding of desiccated thyroid to breeding sparrows is followed by gonadal regression. While a synergistic action of the two glands in their antagonism on the reproductive system may not be definitely established, nevertheless there seems to be evidence of some synergistic action of the glands and a consequent differential inhibition of the pituitary. Whether such an explanation is the reason for observed variations of the animals considered in this report must await further study. Experiments designed to test the veracity of this hypothesis are now in progress.

In conclusion, it remains to be stated that other known effects of adrenalin on the organism do not seem to account for the gonadal responses observed. Thus vasoconstriction might lessen the flow of blood to the reproductive tract, but this is probably too transitory to account for the facts observed. The inhibitory effect of daily injections of adrenalin on smooth muscle might account for the loss of uterine weight, but it would hardly account for the ovarian atrophy obtained. It is difficult to see how the calorogenic action of adrenalin could effect the ovarian changes observed. It is possible that increased heat production plays a role in the male. Finally, hyperglycemia would seem to be stimulatory rather than inhibitory.

SUMMARY

Subcutaneous daily injections of adrenalin given to female rats in suitable dosages were followed by prolonged and repeated periods of diestrus varying from 5 to 40 days. Considerable variation in length of diestrus periods was evident.

Follicular atrophy, "deficiency reactions" in interstitial cells and in luteal cells was observed. Reduction in absolute and relative ovarian weight was obtained.

Reduction in weight and size of the uterus and vagina of treated animals was of common occurrence. Both organs showed atrophic histological changes.

Decrease in pituitary weights of treated animals was evident. Basophiles decreased in number in maximally responding animals. Thyroids of these latter animals were hyperplastic. Elevation of the metabolic rate for 3 weeks after the cessation of injections was observed. Increase in the numbers of insulin producing cells was found in many treated animals.

Possible explanations of the long diestrus periods of certain animals are attempted in view of experimental results obtained and in light of other investigations.

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PLATE 1

EXPLANATION OF FIGURES

- 1 Reproductive tract of control animal sacrificed in diestrus.
- 2 The same of a treated animal littermate after a 40-day injection period.
Figures 1 and 2 are to the same scale, approximately one-half normal size.
- 3 Ovary of the above control animal. 24 $\times$.
- 4 Ovary of treated female showing paucity and atresia of follicles. 24 $\times$.
- 5 Interstitial cells of normal ovary. 340 $\times$.
- 6 Interstitial cells of adrenlin injected animal showing "deficiency reaction."
These cells were observed in ovaries of animals from all litters. 340 $\times$.

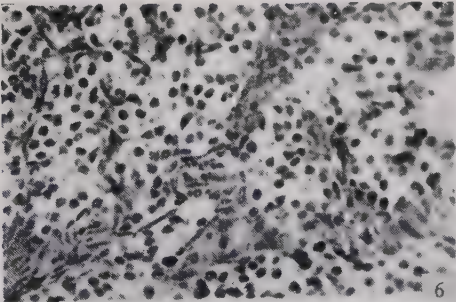
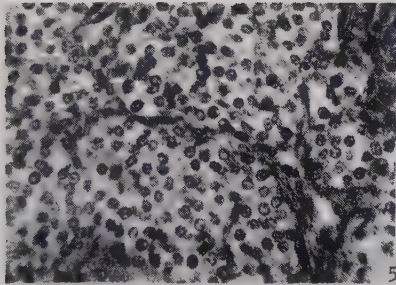
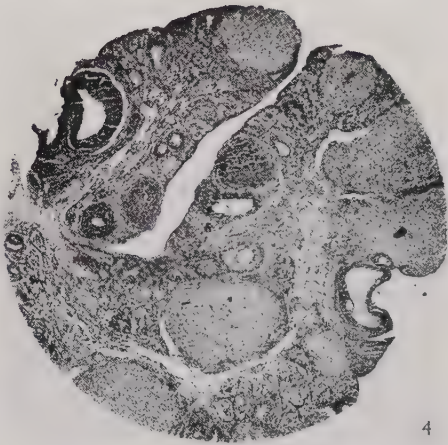
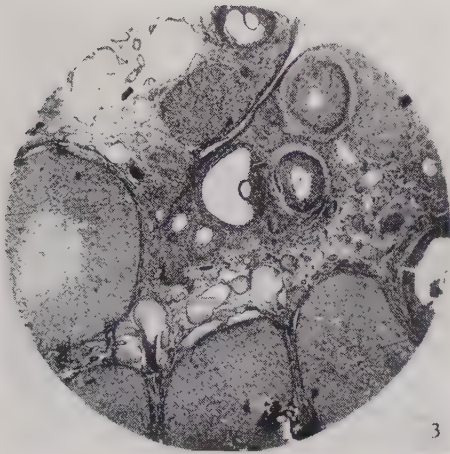
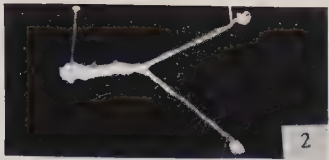
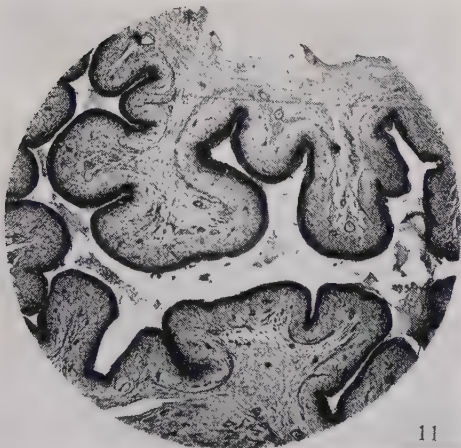
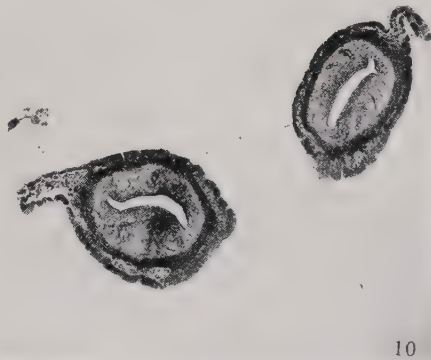
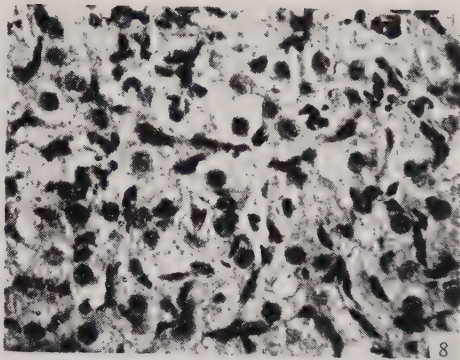
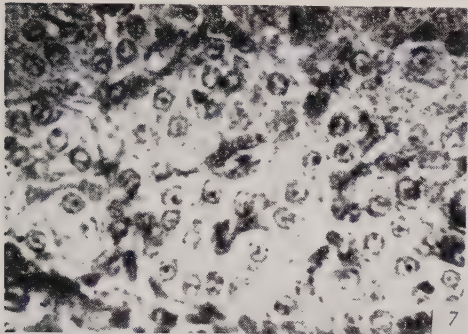


PLATE 2

EXPLANATION OF FIGURES

- 7 Corpus luteum cells of normal ovary. 340 X.
- 8 Corpus luteum cells of adrenlin injected animals showing fibroblast-like cells and cells of pyknotic nuclei, an additional "deficiency reaction" seen in ovaries from all litters. 340 X.
- 9 Cross section of the uterus of a control animal sacrificed in diestrus. 24 X.
- 10 Cross section of the uterus of treated animals showing evident regression. 24 X.
- 11 Cross section of vagina of control rat, sacrificed in diestrus. 24 X.
- 12 Cross section of vagina of treated rat showing tendency of vaginal lumen to close. 24 X.



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ABSTRACTS

In compliance with a renewed request from the Office of Defense Transportation of the Federal Government for the cancellation of conventions and other similar travel-stimulating events, the Executive Committee regretfully voted to cancel the annual December meeting. However, in order to maintain some continuity in the absence of an annual meeting, the Committee decided to continue all feasible society activities and voted in favor of the collection and printing of abstracts as usual.

The following abstracts were received in answer to the usual call for papers issued early in the fall and were prepared for distribution to members of the Society as in the past. The Society is greatly indebted to The Wistar Institute of Anatomy and Biology for the prompt publication and distribution of this material.

ABELMANN, W. H., see Snell et al.

ADAMS, et al. THE THYROTROPIC POTENCY OF THE PARS ANTERIOR OF THYROXIN-INJECTED MICE. A. Elizabeth Adams and Dorothy Jensen, Mount Holyoke College.

Three series of mature, male, albino mice were injected with average total doses of 0.64 mg., 1.32 mg., and 2.66 mg. of thyroxin over 21- and 23-day periods. Controls received a dilute solution of sodium hydroxide in distilled water comparable to the solution in which the thyroxin was prepared or were untreated. At the end of treatment, anterior pituitaries of experimental and control mice were assayed for thyrotropic potency in day-old Rhode Island Red chicks or in a Red-Rock cross. Stimulation was determined by measuring thyroid cell height 24 hours after implanting AP substance.

Anterior pituitaries of normal and injected control mice caused a statistically significant increase in chick thyroid cell height of 70.23% over that of chick controls (untreated or brain-implanted), whereas pituitaries of the three thyroxin-injected series produced an increase of 5.12%, although this also was statistically significant. The difference in stimulation secured indicates a marked decrease in thyrotropin in APs of thyroxin-injected mice. Injection of 0.64 mg. of thyroxin reduced the thyrotropin as strikingly as a dose of 2.66 mg.

Since small increases are statistically significant, it seems possible to utilize a 50% increase rather than 100% (Adams and Beeman, '42) in thyroid cell height of 5-day-old chicks 24 hours after subcutaneous administration of AP substance as the response for a chick unit of thyrotropin. With this unit, APs of control mice in the present experiment contained 963 chick units and those of thyroxin-injected mice 83 chick units of thyrotropin per gram of fresh tissue.

ALLEE, et al. RELATION OF THIAMIN TO SOCIAL AGGRESSIVENESS IN MICE. W. C. Allee and Elizabeth Beeman, The University of Chicago.

Small groups of mice form social hierarchies. In the present study, isolated adult male black mice (C57) developed social orders when brought together, round-robin fashion, for staged encounters. The order shifts at unpredictable intervals with high and low ranks the most stable. Control and stock mice were fed Fox Chow Checkers. Adding thiamin to the normal diet produced no observed changes in behavior. In different control lots, the alpha mouse maintained position for 54, 32, 19, 8, 22, 27, 37, 15, 31, 24, 12, 7, 36, and 28 (mean 25.1) days respectively.

High ranking individuals in six lots of mice with a known social organization were fed thiamin-free food with all other needed vitamins present and with sufficient calories to increase weight. The experimental period lasted 18 to 25 days after which even added thiamin did not always save the lives of experimental animals. Alpha mice retained rank over subordinates on full diet for 71, 23, 23, 22, 19, and 15 (mean 28.8) days from the beginning of the thiamin-free diet. One such maintained his rank through 24 days of this diet (16% weight loss) and until the experiment ended 47 days later, a total of 71 days. The others often remained dominant until near death. The difference between control and experimental lots lacks statistical significance.

Environmental conditions, handling, heredity, and physiological state help determine social position. In these experiments, psychological state, influenced by previous fighting experience, seems more important in maintaining social rank than are those factors affected by thiamin deficiency until advanced avitaminosis is reached.

ANDREW. AGE DIFFERENCES IN THE SUBMANDIBULAR GLAND OF WISTAR INSTITUTE RATS. Warren Andrew, Medical School, Southwestern Medical Foundation, Dallas, Texas.¹

The submandibular glands of forty Wistar Institute rats have been studied, using routine hematoxylin and eosin staining. This gland in rats consists of two distinct kinds of lobules. One type is composed of alveoli which are apparently mucous. The other type consists of alveoli which appear to be of a serous type, although dissimilar from those of the parotid gland.

Age differences in this gland, although relatively slight, are great enough to allow one to distinguish sections from senile animals and those from younger. The most conspicuous differences are in the salivary ducts of the modified serous

¹Part of a study on aging organized by Dr. E. J. Farris of The Wistar Institute with the aid of cooperating scientists and support of the Samuel S. Fels Fund.

type of lobule. In the young animals these ducts show sharply defined lumina. The nuclei are most frequently near the center of the cell. The striations in the cytoplasm are conspicuous. In the mucous lobules these characteristics seem to be retained throughout life. In the serous lobules of middle-aged rats many ducts have their nuclei basally located. The lumina have less sharply defined borders, and striations are harder to distinguish.

In the senile rats the lumina are obliterated in almost all salivary ducts, being filled in with an eosin-staining secretion. Striations are completely lacking in most ducts. The nuclei are almost universally in the basal portions of the cells and often have become flattened or otherwise altered.

BABCOCK, et al. RETARDATION OF BODY WEIGHT INCREASE IN IMMATURE MALE MICE WITH STILBESTROL. George Babcock, Jr., and J. H. Leatham, Rutgers University.

Body weight increase in young rats is definitely retarded by stilbestrol but little consideration has been given to this effect in the mouse with particular reference to dosage. To test the hormone effect, normal male mice, 24-26 days of age, were injected subcutaneously with either 1, 2, 10 or 50 γ daily dosages of stilbestrol in 0.05 cc. oil over a 16-day period. Comparison was made with untreated littermate controls, caged separately. The 1 γ dose was without effect but other dosages suppressed body weight in proportion to the amount administered. Mice receiving 50 γ of stilbestrol daily gained 4.3 gm. in 16 days as compared with the gain of 8.6 gm. in normal littermate controls. The administration of oil alone had no effect on body weight. Furthermore, body weight increase was not retarded when the 800- γ total dosage was given as a single subcutaneous injection and the mice killed after 16 days. Mice receiving 2 γ of stilbestrol daily for 16 days required a 15-day period following cessation of injections for the animals to reach their normal body weight. Approximately the same length of time effected a recovery of body weight from the 50- γ dosage.

BAILEY, P. L. see Treat

BEAMS, et al. DETERMINATION OF POLARITY IN THE POLLEN GRAINS OF VINCA ROSEA BY MEANS OF CENTRIFUGING. Harold W. Beams and Robert L. King, State University of Iowa.

Widely scattered pollen grains of *Vinca rosea* were embedded in an agar-sugar medium having a pH of 7.2 and centrifuged in the dark at 1500 to 20,000 times gravity until the tubes appeared. Under these conditions 60 to 80% of the tubes were observed to arise on the centrifugal side of the grains. Since the grains were not free to rotate in the medium, here as in the pollen grains of the snapdragon (Beams, '43) centrifuging has a marked effect upon the polarity of the grains.

Other physical-chemical factors, such as moisture, seem to affect the polarity of the pollen grains of *Vinca* while indole-3-acetic acid does not.

If a pistil of the snapdragon be placed on the culture medium where the pollen grains of the same plant have been sown, a high percentage of the tubes grow out on the side of the grains toward the pistil. Whether or not this is due to a specific diffusible substance from the pistil or to some other factor is at present unknown.

BEAMS, et al. NEGATIVE "GROUP EFFECT" IN AN ACIDIFIED MEDIUM OF THE POLLEN GRAINS OF VINCA ROSEA. Harold W. Beams and Robert L. King, The State University of Iowa.

Pollen grains of *Vinca rosea* (Periwinkle) were placed in groups of fifty or more on an agar-sugar medium buffered at pH 4, 6.2, 6.8, 7.2, 7.8 and allowed to germinate in the dark. Germination of the pollen grains was inhibited at pH 4. The point of origin of the pollen tubes in the peripherally located grains was noted. At pH 6.2, 6.8, 7.2, and 7.8, 60 to 80% of the pollen tubes formed on the side of the cells away from the mass. Thus, unlike *Fucus* eggs (Whitaker, '37) the pollen grains show a negative "group effect" in an acidified as well as in an alkaline medium. Furthermore, efforts to affect the polarity of pollen grains by germinating them exposed to a gradient between pH 4 and pH 7.2 failed even though a marked inhibition of growth of the tubes was noted near the acidified pole. Under this condition, too, the pollen grains behave differently from *Fucus* eggs (Whitaker, '36) which germinate toward the acid.

BEEMAN, E., see Allee et al.

BEEERS. SOME FACTORS AFFECTING EXCYSTMENT IN THE CILIATE *TILLINA MAGNA*. C. D. Beers, Stanford University and The University of North Carolina.

Permanent resting cysts were obtained upon depletion of the food supply (*Pseudomonas fluorescens*) in lettuce-infusion cultures. Such cysts (11,390 in all) were removed to various solutions whose excysting capacity was under investigation (always at 20°C.), and the mean excystment time was computed for each solution.

Distilled water proved to be a highly effective excysting agent. In a typical experiment with 500 cysts, 498 activated (mean excystment time, 2.86 hours). Mere dilution of the old encystment medium with water was effective, the percentage of excystment depending on the degree of dilution.

The following concentrations of aqueous plant infusions and organic reagents induced practically 100% excystment more rapidly than water, as the mean excystment times show: 0.05% lettuce, 2.48 hours; 0.05% timothy, 2.43; 0.05% Elodea, 2.35; 0.05% alfalfa, 2.23; 0.01% Na acetate, 2.58; 0.005% K citrate, 2.45; 0.025% dextrose, 2.38; 0.025% glycine, 2.36. Increasing the concentration lengthened the excystment time and reduced the percentage of excystment; decreasing it yielded results identical with those obtained with water.

Dilute solutions of urea, yeast extract, ethyl alcohol, Na sulfate, Na bicarbonate and modified Osterhout medium produced excystment, but none was more effective than distilled water; concentrated solutions retarded excystment.

The results indicate that the primary excysting factor is of an osmotic nature, involving the entrance of water into the cyst. A secondary factor concerns the action of specific excystment-inducing substances, among which are glycine and, as found by Haagen-Smit and Thimann ('38) working on *Colpoda*, plant infusions, dextrose and salts of plant acids.

BLIVAISS, BEN B., see Domm et al.

BODINE, et al. THE EFFECT OF HEAT ON PROTYROSINASE ACTIVATED BY DIFFERENT DETERGENTS IN THE PRESENCE AND ABSENCE OF PHOSPHATE. Joseph Hall Bodine and David Leal Hill, State University of Iowa.

Studies on the effect of detergents on the heat activation (65° – 80° C.) of the enzyme, protyrosinase, have shown first, a variation in the character of the tyrosinase formed depending on the detergent used, and second, a variation in enzyme activity depending on the presence or absence of phosphate. Sodium dodecyl sulfate, either alone or in the presence of phosphate, caused a marked increase in activity over that produced with heat alone. The activity resulting from heat in the presence of sodium oleate and phosphate was greater than for heat alone but without phosphate complete inhibition of activity was obtained. In the case of aerosol OT 100% complete inhibition of activity occurred with or without phosphate except at 65° C. where a small amount of activity in the presence of phosphate was observed. All three detergents are activators per se at room temperature.

An explanation of the factors producing these differences in the physical properties of the tyrosinases appears fundamental to an understanding of the activation mechanism of this enzyme.

BODINE, et al. EFFECT OF TEMPERATURE ON THE ENZYME TYROSINASE. Joseph Hall Bodine, Theodore Newton Tahmisian and David Leal Hill, State University of Iowa.

The effect of temperature upon the injury, inhibition and activation of the enzyme, protyrosinase, has been studied in some detail. Enzyme preparations without the addition of buffers (PO_4 or imidazole) seem more susceptible to temperature than when the buffers are present.

Heat activated enzyme (tyrosinase) is more resistant to higher temperatures than enzymes produced by other activating reagents.

Fundamental changes produced during the activation of protyrosinase seem of importance in determining the physical chemical properties of the active enzyme produced.

BODINE, J. H., see Hill.

BRAGG. HABITS IN THE ECOLOGICAL DISTRIBUTION OF OKLAHOMA SALIENTIA. Arthur N. Bragg, University of Oklahoma.

Field studies indicate that feeding habits are of little influence in ecological distribution: breeding habits are of considerable significance. Grassland-limited species have a xeric breeding pattern characterized by lack of a well defined breeding season with the initiation of breeding controlled by rainfall. Woodland and Savannah-limited species have a mesic breeding pattern characterized by the presence of a well defined breeding season after which rainfall no longer stimulates breeding behavior. With a few exceptions, ecologically unrestricted species show a mixed pattern of breeding. Since Amphibia are intermediate between fully aquatic and fully terrestrial vertebrates, one would expect the mesic pattern to

be the primitive; the xeric, the derived one. On this assumption, the facts warrant the deduction that a progressively more xeric environment to the west has limited the dispersal of eastern frogs and toads except as they gradually adopted the traits of the xeric pattern. Once this xeric pattern became fixed in heredity, xerically adapted forms tended to remain in the xeric environment and still mesically adapted forms were halted in dispersal. Mesically adapted frogs, however, have spread westward into the xeric grasslands by utilizing "islands" of mesic communities (ponds, stream-banks, etc.). Hence their ecological distribution is a different thing from their geographical distribution. A bullfrog and a grassland-limited toad, for example, may live in radically different ecological situations within a few rods of each other.

BRAMBEL, et al. MACRO-ORGANISMS AS FISH FOOD IN AND ON THE BOTTOM OF BACK AND MIDDLE RIVERS NEAR BALTIMORE. C. E. Brambel and R. P. Cowles, The Johns Hopkins University.

Thirty years ago, both rivers had an abundant supply of fish. Since that time the number of fish caught in these rivers has decreased somewhat but specially so in the upper reaches of Back River. While the direct action of pollution and other chemical changes may be responsible for the marked decrease in the Back River fisheries, the indications are that the decrease of food, probably due to pollution, in and on the bottom of the river is a factor.

Quantitative samples of the bottom of Middle River, off Clark Point well up the river showed numerous amphipods, some isopods and some molluscs; at the mouth of Middle River a similar condition was found. In Back River, near the mouth numerous amphipods and not much else appeared in the sample; off Stansbury Point, about half way up the river, numerous chironomus larvae, a few small gasteropods, a few pea clams, no amphipods or isopods were taken; in the region of the disposal plant at the head of the river a few chironomus larvae and no other macro-organisms were found.

Each quantitative sample covered an area of 80 sq. cm. of the bottom and extended into the bottom to a depth of 20 cm. By "numerous" is meant 8 to 10, by "some" 5 to 7 and by "few" 1 to 4 specimens.

BROOKS. VERTICAL DISTRIBUTION AND MIGRATION OF PELAGIC DAPHNIA. John L. Brooks, Yale University. (Introduced by G. Evelyn Hutchinson).

A study is being made of the vertical distribution and diurnal migration of pelagic races of *Daphnia* in Connecticut lakes. Woltereck's suggestion that the helmet functions by confining its bearer to a narrow stratum of water, preventing or reducing vertical migration, will not explain the observed facts. Races of *Daphnia* with slight crests may be restricted to as narrow strata of water as ones with well developed helmets, while in other lakes both forms may show an extended vertical distribution. In two cases in which the helmetted adults remain in a narrow zone throughout the day and night, the immature forms with helmets of the same relative size as those of the adults show a pronounced diurnal migration. As both helmetted and non-helmetted *Daphnia* show similar variations in migratory behavior, it seems unlikely that the helmet has any fundamental effect thereon.

In the hypolimnion of two lakes there dwell populations of a small *D. longispina*, all members of which bear a spine on their heads very similar to the spines appearing on the *Daphnia* which Coker and Addlestone (Jour. Elisha Mitchell Sci. Soc. 54: 45-75, 1938) found only in cultures at temperatures above 11°C. However, the temperature of the stratum to which these animals were confined was below 7.5°C., indicating that a minimum temperature of 11°C. is not necessarily critical for spine formation in all races of *Daphnia longispina*.

CAMERON. ATTEMPTS AT PROLONGING THE SURVIVAL TIME OF NEWBORN RODENTS IN CO². John A. Cameron, University of Missouri.

Newborn rodents have a long survival time in high concentration of CO. This time decreases by definite, discontinuous, steps as the age of the animals increases until the adults' survival time is reached at about 3 weeks of age. Three methods were tried in an effort to return newborn animals to a possible state of even greater resistance to CO which may have existed shortly before birth: (1) Newborn rats were subjected to brain transection with an electric cautery, and tested 12 hours later against litter mate controls; (2) Newborn rats and rabbits were injected with morphine sulphate and tested against litter mate controls; (3) Newborn rats and rabbits were chilled to about 65°F. body temperature and tested against litter mate controls. Each of these methods had previously been found effective in returning older individuals to degrees of resistance appropriate to a more juvenile state, but none of the three produced an increase in survival time in the newborn animals. Various reports have described high resistance to anoxia in rodent and other mammalian fetuses surgically delivered prior to term as compared with newborns. In the present work, however, the means employed did not cause the natal threshold of resistance to CO to be re-crossed and no return to the physiological state characteristic of late prenatal life was obtained.

¹Supported by a grant from the National Society of Sigma Xi.

CHARIPPER, H. A., see Finkelstein et al.

CHARIPPER, H. A., see Gordon et al.

COLLYER, P. W., see Richards et al.

COSGROVE, W. B., see Hall, R. P., et al.

COSTELLO, et al. ULTRA-VIOLET PHOTOMICROSCOPY OF NEREIS AND ASTERIAS EGG. D. P. Costello and G. I. Lavin, University of North Carolina and the Rockefeller Institute for Medical Research.

Lavin and Pollister ('42) described a simplified quartz microscope with cobalt and nickel sulphate (liquid) filter utilizing the mercury resonance line 2537 Å. This instrument was used to localize the ultra-violet absorbing components of the eggs of the annelid *Nereis*, and of the starfish *Asterias*. For *Nereis*, photomicrographs were obtained of fertilized and unfertilized eggs, eggs from which the vitelline

membranes had been removed, and strongly centrifuged eggs, all compressed under a quartz coverslip. The jelly layer (of the fertilized egg) and the vitelline membrane (before or after fertilization) absorb a negligible amount of U. V. λ 2537 Å. The principal absorption is by the yolk spheres of the cytoplasm (demonstrated in the centrifuged eggs); a lesser amount by the hyaline cytoplasm, and still less by the contents of the germinal vesicle (oöcyte nucleus). The nucleolus absorbs very little, and the oil droplets show complete transmission.

For *Asterias*, studies were made only of the compressed unfertilized egg, in the germinal vesicle stage, before and after centrifuging. The external jelly and vitelline membrane absorb very little. The principal absorption is by the yolk granules, less by the hyaline protoplasm and rather little by the nuclear contents of the germinal vesicle. There is, however, a striking difference in the U. V. absorption capacity of the nucleolus of the *Asterias* egg as compared with that of the *Nereis* egg. Whereas the nucleolus of the *Nereis* egg is quite transparent to this wave-length of U. V., the nucleolus of the *Asterias* egg absorbs about as well as the yolk zone.

COWLES, R. P., see Brambel et al.

DAVIS, M. B., see Park et al. 1 and 2.

DOMM, et al. MALE COPULATORY BEHAVIOR IN BROWN LEGHORN HENS FOLLOWING IMPLANTATION OF TESTOSTERONE PROPIONATE PELLETS. L. V. Domm and Ben B. Blivaiss, Whitman Laboratory of Experimental Zoology, The University of Chicago.

Previous experiments had shown that daily injections of testosterone propionate in pullets for periods up to 7 months failed to induce male copulatory behavior though crowing and masculine courtship activity were observed.

Observations were continued on four pullets (A, BW, G, GA) receiving testosterone propionate pellets (average weight 33.4 mg.) when 5 months old and at 84, 164, and 244 days thereafter. A was first observed crowing on 34th, grab and attempt mounting on 95th, grab, mount and copulate on 110th, and waltz and circle on 114th day. BW crowed on 34th, chased on 95th, mounted and copulated on 103rd, and waltzed and circled on 127th. G crowed on 32nd, chased on 96th, and waltzed and circled on 110th. GA crowed on 32nd, chased on 96th, and waltzed and circled on 103rd. G and GA squatted and received copulation until 154th day, thereafter avoiding male. Behavior patterns once established generally persisted though crowing ceased in all 66 days after second implantation and was resumed 6 days after third implantation in G and GA, and 11 days after in A and BW.

One of treading pullets (A) never laid while the other (BW) laid six eggs between 69th and 80th day following second implantation. One of non-treading pullets (G) laid six eggs between 57th and 74th and the other (GA) one egg on 79th day after second implantation. Two controls receiving cholesterol pellets laid from 51st and 52nd days after second implantation until 25th and 43rd days respectively after third implantation.

DOMM, L. V., see Taber et al.

DURY, A., see Fraps et al.

EASTLICK. REPLACEMENT OF MUSCLE BY ADIPOSE TISSUE IN TRANSPLANTED EMBRYONIC LIMBS OF THE CHICK.¹ Herbert L. Eastlick, Washington State College.

This report is based on an histological study of 130 grafts 3 to 21 days of age. A few embryonic fat cells were observed in the subcutaneous tissue of 9-day-old grafts but none were seen in areas where muscle fibers were being differentiated. Differentiation of the muscle ceased in 10- to 13-day grafts and the fibers were destroyed, apparently by phagocytosis and sarcolysis. However, a rather ramified network of capillaries persisted. Clusters of pre-fat cells developed along these. Most of them were round or oval although some possessed one or more short processes. The nuclei were 5 to 6 μ . in diameter, filling the major portion of each cell. The chromatin was coarser and distributed more irregularly than in fibroblasts. It is suggested, that the pre-fat cells arise from undifferentiated cells which occur in close proximity to the blood vessels.

Small fat globules began to appear in the cytoplasm of the fat cells in 10- and 11-day grafts. In most instances the nuclei remained in the center of the cells and so became surrounded with fat droplets. Such cells resembled rather closely those which have been described in the so-called hibernating gland of mammals. The fat globules in these multilocular cells began to coalesce in 13- to 15-day specimens and by the 21st day the extensive areas of adipose tissue were composed primarily of cells of the typical unilocular type.

¹ Aided by a grant from the American Philosophical Society.

EISENBRANDT. VARIATION IN THE PH OF HUMAN SALIVA. L. L. Eisenbrandt, University of Kansas City.

The pH of saliva of seven subjects has been studied statistically. During a period of 1 year 1552 tests were made at 9 and 11 A. M. and 1, 3, and 5 P. M. daily for 1 week each month. The unstimulated saliva was tested with the glass electrode within 1 minute after deposition into the sample cup.

The mean pH of saliva for the group was 6.64 ± 0.006 (std. error). The group varied significantly from month to month. The range of monthly pH means was 6.53 to 6.72. There were two high points during the year, fall and spring; the two low points were mid-winter and early summer. The group was significantly variable at the bi-hourly periods during the day. The low mean pH was 6.55 at 9 A. M. followed by increases to the high point, 6.71, at 5 P. M. The subjects were significantly variable from one another for the year. The pH range was 6.52 to 6.77. The monthly means for each bi-hourly period were found to vary significantly during the year, however, the relationship of one period of the day with another remained remarkably constant.

This work indicates that an individual has his own characteristic pH range of saliva, that each bi-hourly period has its characteristic range, that the pH of saliva increases during the day and that there is evidence of seasonal variations.

ENGLE, et al. SEASON OF ATTACHMENT OF MUSSEL LARVAE. James B. Engle and Victor L. Loosanoff, U. S. Fish and Wildlife Service, Milford Biological Laboratory.

Observations on the attachment or setting of mussel larvae, *M. edulis*, carried on in Milford Harbor in 1942 and 1943 showed that the setting season extended from the early part of June to the end of August. The period of intense setting occurred from the middle of June to the middle of July. The peak of setting each year was confined to 1 week — from June 29 to July 6.

Mussels set in large numbers on the collectors kept on the bottom, at a depth of 6 feet, and also on the collectors suspended at the mean low water level. Collectors kept at +2 and +5-foot tidal levels showed an extremely light set. The number of mussels found in different sections of the vertical collectors showed that the upper limit of heavy mussel setting lies between 1 and 2 feet above the mean low water mark. Above that level the intensity of setting diminished abruptly. Between that level and the bottom the mussels set in large numbers. Observations indicated that setting of mussel larvae usually occurs when the water level is near the mean low water mark. The failure of mussels to set toward the end of the summer might be ascribed to the absence of mature larvae. Although spawning continued at that time and young larvae were present in the water, virtually all of them disappeared before reaching the advanced stage of development.

ENGLE, J. B., see Loosanoff et al.

ELIAS. CAUSE FOR BLUE, GREEN, AND RED COLOUR IN ANURA. Hans Elias, Middlesex University

Eberth (1869) described guanophores of *Rana esculenta* and *temporaria* appearing blue or purple in transmitted light, shining orange or blue in reflected light. This observation, though confirmed by Hadjioloff ('29), was rejected by the majority including myself. Blue and green colour were attributed to the cloudy medium phenomenon. Red colour was believed to be caused always by true erythrophores: cells containing really red granules. New observations, however, show that the phenomenon described by Eberth is true for four American species:

Some guanophores of *Rana pipiens* and *clamitans* appear blue in transmitted and copper-red in reflected light, others red or purple in transmitted, but blue in reflected light. *Rana palustris* has guanophores, brown, pink, or purple in transmitted light which appear red, blue-green, or blue in reflected light, according to the angle of incidence. *Rana sylvatica* has guanophores blue in transmitted, yellow in reflected light, and others purple in transmitted, blue in reflected light.

The physical and chemical reasons for this phenomenon, similar to fluorescence, are now under investigation in this laboratory.

As in *Bufo viridis* (Goubeaud, '31), *Rana temporaria* (Schmidt, '19) and *latastei* (Elias, '37), also in *Rana sylvatica*, *Bufo americanus* and *fowleri* (new observations) red is produced by true erythrophores containing really red granules. But in *Rana pipiens* and *palustris* red is produced by red-reflecting guanophores.

Green arises usually through xanthophores overlying blue-reflecting guanophores, but in *Bufo americanus* through melanophores alternating with yellow xantholeucosomes.

EVANS. EFFECTS OF ROENTGEN RADIATION ON CATALASE EXTRACTED FROM ARBACIA SPERM. T. C. Evans, College of Physicians and Surgeons, Columbia University.

Catalase activity was detected in extracts of *Arbacia punctulata* sperm prepared as follows. Sperm were collected in sea water, packed by centrifugation, and the sea water replaced by distilled water in proportions of 1 part packed sperm to 10 parts water. The sperm were then stirred in the water and allowed to stand for 30 minutes at 15–20°C. The material was then filtered and the activity of the filtrate determined by adding different amounts to hydrogen peroxide in Warburg manometers at 25°C.

Under proper conditions, the catalase material greatly reduced or even completely removed the toxicity of heavily irradiated water (or of 1:100,000 H_2O_2) to *Arbacia* sperm.

The catalase activity of the extract was reduced by roentgen irradiation. The amount of radiation destruction could be decreased by (1) decreasing the dosage, (2) increasing the concentration of the enzyme during irradiation, and (3) adding protective substances such as egg albumin.

EVANS. ROENTGEN RADIATION DISINTEGRATION OF MNEMIOPSIS (COMB JELLY).

T. C. Evans, College of Physicians and Surgeons, Columbia University.

During the past summer at the Marine Biological Laboratory it was observed that roentgen irradiation of *Mnemiopsis* resulted in its immediate disintegration. A dosage of only 1200 roentgens was required to produce shrinking and loss of turgidity. Partial disintegration (comb bands still attached at aboral tip and still capable of luminescence) was produced by an irradiation of 2400 r, and 4800 to 16,800 r was sufficient radiation to cause complete disintegration of even the largest and most vigorous specimens. The injury does not appear to be an autotomous reaction. Even though the exact mechanism is not clear, it is of interest to note that this organism is relatively radiosensitive and that there is an apparent similarity between this reaction and that of roentgen dispersion of the jelly that surrounds the egg of *Arbacia*, *Asterias*, and *Nereis*.

FANKHAUSER, G., see Humphrey et al.

FINKELSTEIN, et al. INFLUENCE OF CASTRATION, TESTOSTERONE, AND COBALT ON RED CELL REGENERATION IN ANEMIC RATS. Grace Finkelstein, Albert S. Gordon, and Harry A. Charipper, Department of Biology, Washington Square College of Arts and Science, New York University.

Four to 5 cc. of blood were withdrawn by cardiac puncture from adult male and female rats 1 week after castration. The red cell counts dropped from normal values of 7.2–9.2 to 4.1–5.2 millions/cu. mm. on the day following the bleeding. Normal counts were attained in the castrated males after 57–69 days and in the females after 34–48 days. It has been shown that normal unoperated males exhibit a greater rate of red cell regeneration following a bleeding than do normal females (Gordon and Charipper, '43). The fact that castration accelerates regeneration in the female and depresses it in the male constitutes additional experimental evidence for the hypothesis that the male and female sex hormones exert opposite effects on the erythropoietic process.

In another experiment, a comparison of the effects of testosterone and cobalt on erythropoiesis was made in three groups of normal adult male and female rats. Approximately 10 cc. of blood were withdrawn from all animals over a 2-day period. On the third day the counts had dropped from values of 7.4-9.4 to 3.9-4.6 millions/cu. mm. One group was left untreated. A second received daily injections of 1.25 mg. testosterone propionate (an optimal dose). A third group received daily injections of a previously determined optimal dose of 2.5 mg. $\text{CO}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$. Both testosterone and cobalt were found to accelerate attainment of normal red cell count levels in the bled animals. Cobalt displayed a greater power than testosterone in accelerating red cell regeneration in the anemic male rat. In the female, its effect was approximately equal to that exerted by testosterone.

FINLEY. SOME EFFECTS OF ULTRA-VIOLET UPON FLECKING IN THE DOMESTIC PIGEON. Harold E. Finley, Morehouse College.

Flight feathers were plucked from barred and barless "ash-red" male domestic pigeons and the bare areas of the skin exposed immediately to ultra-violet radiations. The object of the investigation was to determine whether flecking could be either induced or increased by means of the treatment administered. The source of the ultra-violet radiations was a Hanovia analytic model lamp equipped with a Hanovia filter Sc 5024. The treating distance was 19 cm. The time of exposure was 3 minutes. It appears that the radiations induce a very slight and temporary increase in the amount of flecking.

FINLEY, H. E., see Miller

FRAPS, et al. RELATIVE EFFECTIVENESS OF VARIOUS GONADOTROPES IN THE INDUCTION OF OVULATION IN THE HEN (*GALLUS DOMESTICUS*). R. M. Fraps and A. Dury. Bureau of Animal Industry, U. S. Dept. of Agriculture, Beltsville, Md.

Determinations have been made of the relative effectiveness of a luteinizing preparation (LH) from sheep pituitaries, a follicle stimulating preparation (FSH) also from sheep pituitaries, a pregnancy urine (PU) extract and pregnant mares' serum (PMS) in forcing ovulation in the hen. The LH and FSH preparations were furnished through the courtesy of Drs. Schooley and Bates of the Difeo Laboratories. Antuitrin-S (Parke, Davis & Co.) and Gonadin Serum (Cutter Laboratories) served as PU extract and PMS respectively.

All tests were carried out for effect on first follicles of recurrent clutch sequences at 6 to 8 hours prematurity. The quantity of material (weight or independently established units) administered intravenously into each hen and inducing ovulation in 50% of ten or more injected hens was taken as the basis of comparison of the several gonadotropes.

The LH preparation was effective at 0.05 mg., or about 0.2 R. U., the rat unit being based on increase in seminal vesicle weight in normal 21-day-old animals. Approximately 1.5 mg. of the FSH preparation was required, a quantity large enough to carry appreciable LH. Around 250 I. U. of the PU extract and 20 to 25 R. U. of PMS were also effective.

In summary, ovulation in the hen is much more effectively induced by the LH component of sheep anterior pituitary than by FSH of the same origin or by gonadotropes of extra-pituitary origin.

FRAPS. POTENCIES OF ANTERIOR PITUITARY GLANDS OF MATURE CHICKENS IN THE INDUCTION OF OVULATION IN THE HEN. R. M. Fraps. Bureau of Animal Industry, U. S. Dept. of Agriculture, Beltsville, Md.

Comparison was made of the effectiveness of anterior pituitary glands from normally laying hens, from hens with resting ovaries, and from cocks in the induction of ovulation in the hen.

Glands were removed immediately following sacrifice of donors, weighed and prepared as suspensions in distilled water. The suspensions were injected intravenously for effect on first follicles of clutch sequences at 6 to 8 hours pre-maturity. The quantity of tissue administered to each hen and causing ovulation in approximately 50% of ten or more injected hens served to define the test unit.

Anterior pituitaries from laying hens contained about 3.3 units per milligram fresh tissue, from hens with resting ovaries 5.0 units per milligram, and from cocks close to 40.0 units per milligram. On the basis of dry weights, 1 mg. of tissue from laying hens contains about 17 ovulation units, from hens with resting ovaries 25 units, and from cocks some 200 units.

The potencies of chicken pituitaries, particularly those of the male, are high in comparison with values yielded by mammalian pituitaries and pituitary preparations thus far tested similarly in the hen, although no correspondingly notable effectiveness of avian pituitaries appears to have been reported in mammalian responses. The question of species specificity of the hormones of the anterior pituitary may possibly be involved in these considerations.

FRASER. STUDIES ON THE MUTANT FACTOR RHINO IN THE MOUSE, AND ITS INTERACTION WITH THE NAKED AND HAIRLESS MUTATIONS. F. Clarke Fraser, McGill University. (Introduced by Arthur G. Steinberg.)

The recessive factor rhino (hr^{rh}) causes a hyperplasia of the epidermis and its derivatives, which is manifested as (1) a hyperkeratosis of the epidermis and follicle neck wall, (2) subsequent cyst-formation in the hair-canals (which causes an increase in surface area and consequent wrinkling of the skin), in the sebaceous glands and in the follicle ends, and (3) overgrowth of the nails. Hair loss is considered to result from widening of the hair-canal and a consequent lack of the support supplied by the normally tight-fitting follicle neck when shortening of the follicle occurs. Hairless mice show a similar, but less extreme tendency towards hyperplasia. Hairless/rhino hybrids are intermediate in character. Mice of any of these types which are simultaneously heterozygous for the Naked factor show an exaggeration of the follicular keratosis which may be due to an irritation of the follicle neck wall by the irregular, Naked-type hairs. There are, however, indications of an interaction of the Naked and rhino (or hairless) reaction-chains previous to hair loss. In mice homozygous for the Naked factor the exaggeration is even more extreme.

Transplantation experiments show that rhino skin adjacent to normal skin behaves non-autonomously, indicating that rhino skin is able to utilize, but unable

to produce, some substance necessary for the maintenance of normal stratified squamous epithelium. Results of vitamin A treatment, although complicated by a possible hypervitaminosis, indicate that the rhino and hairless conditions may involve a partial inability to metabolize vitamin A.

GAUNT, R., see Nelson et al. 1

GIESE. STUDIES ON THE RESPIRATION OF YEAST AFTER IRRADIATION WITH ULTRAVIOLET LIGHT. Arthur C. Giese, Stanford University.

The accelerated endogenous respiration of yeast irradiated with ultraviolet light is reduced by azide and cyanide and may be increased by dinitrophenol. The respiratory quotient is about 0.56 compared to the control 0.61. Addition of extracts of cells killed by intense irradiations does not accelerate respiration of controls comparably to ultraviolet radiation though such extracts may have transitory effects such as are obtained from a low concentration of nutrients. The acceleration of respiration may be due to the removal of some factor which prevents the cell from using its reserves.

The reduced exogenous respiration of yeast irradiated with ultraviolet light is not increased by mixtures of the vitamin B complex, indicating that it is probably not due to the breakdown of these ultraviolet absorbing compounds. Glucose is stored to only a small extent in ultraviolet treated cells as compared to controls. The results on both endogenous and exogenous respiration suggest a rather general injury to the respiratory mechanism of the cell.

GIESE, A. C., see Reed

GILBERT. THE ALGAE-EGG RELATIONSHIP IN *AMBYSTOMA MACULATUM*, A CASE OF SYMBIOSIS. Perry W. Gilbert, Cornell University.

It is well known that certain unicellular green algae thrive within the jelly envelopes of *Ambystoma maculatum* eggs. The algae apparently profit by the association for they multiply rapidly and flourish within the egg envelopes. The following experiments indicate that the developing embryos likewise are benefited.

Eight large, newly laid, algae-inhabited bunches, or a total of over 800 *Ambystoma maculatum* eggs, were secured from the breeding ponds. Each bunch was halved, one-half being placed in a lighted battery jar and the corresponding half in a jar kept in the dark. All jars were filled with running water, the temperature of which remained constant. The algae flourished in the lighted egg masses but did not develop in the corresponding masses kept in the dark. By comparing the developmental progress of corresponding algae-inhabited and algae-free masses, the following results were obtained: (1) in general the algae-inhabited eggs either began hatching earlier or hatched over a shorter interval of time, (2) the presence of the algae was associated with a lower mortality rate prior to hatching, (3) newly hatched larvae from algae-inhabited eggs averaged 13.3 mm. in length while those from algae-free masses averaged 12.0 mm. in length, (4) newly hatched larvae from algae-inhabited eggs were approximately

2.3 Harrison stages in advance of larvae emerging at corresponding periods of time from algae-free eggs.

Control experiments indicated that the above results were due to the presence or absence of the algae, not to the presence or absence of light.

GOLDSMITH, E. D., see Gordon et al.

GORDON, et al. EFFECTS OF THIOUREA AND SULFADIAZINE ON THE THYROTROPIC HORMONE CONTENT OF THE PITUITARY GLAND AND BLOOD SERUM OF RATS.¹ Albert S. Gordon, E. D. Goldsmith, and Harry A. Charipper, Department of Biology, Washington Square College of Arts and Science, New York University.

Groups of adult male rats were fed a diet containing 1% thiourea or 1% sulfadiazone for both 14- and 45-day periods. This resulted in marked enlargement of the thyroid gland (Mackenzie and Mackenzie, Astwood et al., '43). The blood sera and pituitaries from these animals were collected and pooled. The pituitaries were minced to a fine pulp and suspended in distilled water. 0.05 cc. quantities of the sera and of the pituitary suspensions (1-100 dilution) from sulfadiazine-treated, thiourea-treated, and normal untreated rats were injected pleuroperitoneally once every 2 days into groups of young *Rana pipiens* tadpoles according to the method of D'Angelo, Gordon, and Charipper, ('42). At frequent intervals, the hind limb lengths of these animals were measured and the times of forelimb extrusion noted. These assay experiments indicated that the sera and hypophyses from the drug-treated rats contained considerably smaller quantities of metamorphosis-inducing substance than those obtained from untreated rats. Histological study of the thyroid glands of the treated tadpoles revealed that these results were due largely, if not entirely, to differences in thyrotropic hormone content of the injected materials.

In view of the experiments of others, these results are best interpreted to mean that thiourea and sulfadiazine, by inhibiting the production of normal thyroid principle, cause an increased release of thyrotropic hormone from the hypophysis into the blood where, however, it appears in reduced amount because of its increased utilization by the enlarged thyroid gland.

¹ This work was supported by a grant administered by the Commonwealth Fund.

GORDON, A. S., see Finkelstein et al.

GRAVES. DEVELOPMENT OF THE GOLDEN HAMSTER, *CRICETUS AURATUS* WATERHOUSE, DURING THE FIRST NINE DAYS. Artis P. Graves, State University of Iowa. (Introduced by Emil Witschi.)

The egg of the hamster differs from that of the rat by its large size and the abundance of coarse yolk granules. These granules undergo metamorphic changes that resemble those described by various authors for marsupials, in some respects.

First decidual reactions begin at 48 hours. Early morula stages are in the uterus at 72 hours consisting of 5 to 12 blastomeres. At this stage subepithelial cells transform into decidual cells. The mucous glands recede and completely disappear by 7½ days. In the rat they persist through the eleventh day.

It is found that the hamster develops faster than any known placental mammal and fully as rapidly as the opossum. In the rat, morula and preimplantation blastocyst have an ovoid shape, with animal poles directed mesometrically. In the hamster they are globular and not specifically oriented. The trophoblastic cone never assumes the sharply pointed shape which is characteristic for rat and mouse. From the end of the sixth to the beginning of the eighth day, the blastocyst becomes ellipsoidal, but by the middle of the eighth day it has returned to a spherical shape.

The inner cell mass of the hamster and the other Muridae contains all of the elements which invaginate in gophers, namely, embryo, amnion, and chorion forming elements, though in a preprimordial condition. Designations like ectoplacental cavity and epamniotic cavity, which indicate the general position, but do not express the morphological value of this cavity should be replaced by the term chorionic cyst.

HALL, E. K., et al. VOLUME AND CELL NUMBER IN THE SPINAL GANGLIA OF THE BRACHIAL PLEXUS. E. K. Hall and M. A. Schneiderhan, Departments of Anatomy and Chemistry, University of Louisville School of Medicine.

In the course of an investigation on the effects of fore limb amputation in fetal stages on the development of the spinal ganglia of the white rat (*Anat. Rec.*, vol. 82, p. 467), data have accumulated on the volume and relative cell number of the normal ganglia of four operated specimens fixed at birth. A total of 30,165 cells were counted and 794 sections measured.

The total cell count for all of the ganglia of the normal brachial plexus (CV to TI) is approximately the same in all four cases (greatest variation 15%). These cells are, however, distributed rather irregularly among the ganglia, the ganglia with the largest number having about 50% more cells than the ganglia with the smallest number. The variations in the volume measurements of the ganglia are greater but more regular, so that the average volume increases steadily from CV to CVIII (increase of 50%), and decreases in TI to a figure between that for CVI and CVII.

The average size of the cells of the CV ganglia appears to be smallest; this increases progressively in the CVI and CVII ganglia and becomes greatest in the CVIII ganglia, where the average cell size is 50% greater than in the CV ganglia; the average size of the cells of the TI ganglia sinks to that of the CVI ganglia.

Like the spinal cord, the spinal ganglia enlarge progressively in the lower cervical region; in this region, ganglion size is correlated with cell size rather than cell number.

HALL, R. P., et al. THE QUESTION OF THE SYNTHESIS OF THIAMIN BY THE CILIATE, GLAUCOMA PRIFORMIS. R. P. Hall and W. B. Cosgrove, New York University.

In attempts to develop suitable basal media for the study of vitamin requirements in ciliates, the writers have tried various protein preparations including a "vitamin-free casein" used previously by Dewey ('41) and by Kidder and Dewey ('42). This casein, with certain added salts, constitutes a satisfactory

culture medium for *Glaucoma piriformis*. Casein heated in alkaline solution for an hour or more no longer supports growth of this ciliate. However, serial transfers are assured by the addition of thiamin to the "dethiaminized" medium, while much better growth is obtained with added thiamin and riboflavin. Addition of the thiazole and pyrimidine components of thiamin to the dethiaminized medium is ineffective, and there are no indications that *G. piriformis* can synthesize thiamin from these substances. The addition of thiazole and pyrimidine to untreated casein medium likewise produces no detectable effects on growth, in contrast to the effect of added thiamin in prolonging the phase of maximal density. Our data afford no evidence that *G. piriformis* is able to synthesize thiamin, either from the thiazole and pyrimidine components of thiamin or from simpler substances.

HARTLEY. EFFECTS OF SEX HORMONES ON THE UROGENITAL ORGANS OF THE GARTER SNAKE EMBRYO. Richard T. Hartley, State University of Iowa. (Introduced by Paul L. Risley.)

In the garter snake, sex differentiation occurs rapidly and directly, during the third week of development, much earlier than in other reptiles. The testis transforms into medulla with seminal tubules and stroma, with little cortical development other than the original germinal epithelium. In the ovary, the medulla is at no time organized into definite testicular components.

Injection of .25 mg. of testosterone propionate into the amniotic cavity during the third week of gestation resulted in increased size of seminal tubules in the testis, and increased numbers of spermatogonia. In female embryos, decreased ovarian cortex, retarded follicle development, and some organization of medulla into sex cords with germ cells followed this treatment. Injection of .03 mg. of estradiol dipropionate into the amniotic cavity during the third week of gestation produced slight decreases in size of seminal tubules, and an increase in stromal tissue of the testis, but did not affect the ovary. Slight effects upon the oviducts were obtained.

Intraperitoneal injections of 1.25 mg. of testosterone propionate into pregnant females on alternate days throughout gestation, after the tenth day caused hypertrophy of the oviducts of the female, but had no marked effect upon the gonads of either sex. Similar treatment with 0.1 mg. of estradiol dipropionate delayed parturition, enlarged segments of, Mullerian ducts in male embryos, and induced marked hypertrophy of Mullerian ducts in female embryos.

The limited effects on the gonads were probably due to the short period of bisexuality, and slight responsive capacities of the reproductive organs.

HERSH. A FURTHER APPLICATION OF THE ALLOMETRIC EQUATION. A. H. Hersh. Western Reserve University.

The allometric equation $y = bx^k$ has been applied to two main types of data. In the first case x and y refer to the differentially growing parts with interpretations mainly in ontogenetic terms. In the second case the x 's refer to the size of some homologous part of a series of related organisms that differ in size, the y 's to another part which is likewise homologous throughout the series. This leads

to the detection of allometric tribes. In the third case, to which the equation has not heretofore been applied, x_1, x_2, x_3 , etc., refer to the magnitude of a series of measurements on one kind of organism and y_1, y_2, y_3 , etc., to the corresponding homologous measurements of a second type of organism. For example, when six skeletal measurements (Castle, '22, Carnegie Inst. Publ. no. 320) of Flemish rabbits (y) are plotted against the homologous series of measurements of Polish rabbits (x) the equation holds with $k=1.11$ and $\log b = -0.0803$. A skeletal measurement in the Flemish race equals that of the Polish raised to the 1.11 power. So far as the data go, none of the genetic differences acts locally on the skeleton. Data on a more exhaustive series of homologous measurements treated in this manner should allow conclusions to be drawn relative to the problem of generally and locally acting genetic factors and provide a more illuminating comparison of the morphological pattern of different related types.

HIESTAND, et al. SOME OBSERVATIONS ON ANDROGEN-TREATED BABY CHICKS.

Wm. A. Hiestand and D. E. Stullken, Purdue University.

Baby chicks injected with androgen were subjected to decompression and survival compared to that of untreated controls. At collapse the birds were slowly recompressed and salvaged. Each chick received 4 injections of 1.0 mg. (testosterone propionate in oil) over a period of 2 weeks beginning at 7 days of age. Each showed the typical androgen effect of comb hyperemia and enlargement, and social dominance (peck order) while young. Peeping sounds common to baby chicks were absent and fewer signs of fright appeared. When decompressed very little distress appeared as compared to untreated controls. Just before collapse untreated controls panted but androgen-treated chicks did not. Collapse occurred at about the same barometric pressure (220 mm. Hg.) in both groups showing that androgen did not significantly alter hypoxic resistance.

The baby chicks definitely demonstrated the androgenic influence on social dominance while young but after the lapse of 1 to 2 months they gradually assumed a social inferiority whereupon they were dominated by the other (non-androgen-treated) birds. In fact, two were killed by the pecking of the non-treated birds. It was also apparent that comb growth of the early androgen-treated birds had lagged far behind that of the others indicating the influence of (1) anti-hormonal effects or more likely (2) suppression of the gonadotropin of the anterior lobe.

HIESTAND, et al. EFFECTS OF AUTONOMIC DRUGS ON COLOR CHANGES IN ANOLIS CAROLINENSIS. Wm. A. Hiestand and D. E. Stullken, Purdue University.

Drugs causing green coloration by contraction of the skin melanophores in *Anolis* are epinephrine, histamine, sparteine, and tyramine. Those causing brown coloration due to expansion of the melanophores are posterior pituitary extract, and epinephrine following ergotoxine.

The sympathetolytic effect of large doses of ergotoxine persists for as long as 13 days during which time the chameleon remains green and turns brown following injection of epinephrine. Immediately after ergotoxine is injected the skin becomes brown and does not turn green upon injection of epinephrine. On

the following day and for some time thereafter the skin remains green and becomes brown upon injections of epinephrine showing the reversal of effects by ergotoxine.

Ephedrine exerts a true sympathomimetic effect with more than usual expulsion of the orange sexual skin of the ventral neck. The sympathomimetic effects of sparteine and tyramine are very transient.

HIESTAND, et al. RELATIONSHIP OF RATE OF DECOMPRESSION TO DEGREE OF HYPOXIC SURVIVAL. Wm. A. Hiestand and Helen M. Rogers, Purdue University.

It is generally believed that a greater degree of hypoxia can be tolerated with rapid than with slow decompression and that therefore a higher altitude can be reached upon rapid ascent than upon slow ascent. This is true with mice only if the rate of ascent (decompression) is so rapid that a high altitude is reached before the individual has had time to become severely hypoxic.

A total of 62 white mice of the Hygienic Strain all of approximately the same age and weight were subjected to the effects of decompression. A total of 30 were decompressed at the rapid rate of 58.8 mm. Hg. per minute average until dead. A total of 32 were decompressed slowly at the rate of 12.4 mm. Hg. per minute average until dead.

The mean death point for the first group (rapid decompression) was 174.2 mm. Hg. (equivalent to 35,535 feet altitude). The mean death point of the second group (slow decompression) was 152.2 mm. Hg. (equivalent to 38,362 feet altitude). The possibility of CO₂ accumulation was considered. When CO₂ was removed with soda-lime a slightly greater tolerance resulted. The ability of the mouse to hyperventilate its lungs no doubt is important but it seems apparent that increasing the time for acclimatization involves other factors than elimination of CO₂ in increasing the animal's tolerance to hypoxic conditions.

HIESTAND, W. A., see Noland

HIESTAND, W. A., see Stullken et al.

HILL. CERTAIN RELATIONSHIPS BETWEEN CARBOHYDRATE AND RESPIRATORY METABOLISM DURING EMBRYONIC DEVELOPMENT (ORTHOPTERA). David Leal Hill, State University of Iowa. (Introduced by Joseph H. Bodine.)

Analysis of the developmental changes in concentration of free and combined reducing substances and their partition between whole egg and embryo in the grasshopper, *Melanoplus differentialis*, indicates a general agreement with Needham's theory of the sequence in embryonic energy sources.

In early prediapause a rapid decrease in the amount of free reducing materials, associated with high respiratory quotients, showed an initial carbohydrate utilization. Significant also is a parallel decrease in pyrophosphate during this time.

Synthetic processes in the egg during later development made difficult a correlation of the data with energy requirements. Losses in reducing substances such as would be associated with further carbohydrate oxidation could not be demonstrated. Indirect evidence points to an intermediate phase of protein utilization. The changes in concentration of reducing substances in post diapause were

apparently those associated with growth and differentiation of the embryo and could not be ascribed to energy metabolism. The low respiratory quotient and marked decrease in fatty acid content demonstrate that fat is the predominate energy source in late development.

From the seventh to the ninth days in prediapause, during which time cuticle is deposited, it was possible to show a conversion of fat to carbohydrate on the basis of changes in the metabolites concerned.

HILL, D. L., see Bodine et al. 1 and 2

HINRICHS. NUCLEAR FRACTIONATION OF POLYMORPHS FOLLOWING PHYSICAL FATIGUE OF ATHLETES. Marie A. Hinrichs, Southern Illinois Normal University, Carbondale, Illinois.

Blood smears on thirty-six women and forty-one men athletes were obtained immediately before and immediately after fatiguing exercise, and were stained with Wright's stain and buffered. Studies of the degree of nuclear fractionation revealed that in 47% of the cases a relatively significant increase appeared in the number of nuclear segments in post-exercise smears as compared with those in control smears. Of the remainder, 31% showed no significant change, while 22% showed a decrease in the number of nuclear divisions.

The median values for pre-exercise nuclear divisions were 4.18 (men) and 4.28 (women) as compared with post-exercise values of 4.33 (men) and 4.52 (women). Since it had previously been noted that fatiguing exercise also caused a significant decrease in the relative percent of polymorphonuclear leucocytes in a differential count, (Hinrichs¹) it would appear that in most cases the younger polymorphs are not entering the peripheral circulation as fast as the old ones are destroyed.

¹ Hinrichs, M. A. Proc., Soc. Exp. Biol. and Med. Vol. 45, p. 685, 1940.

HOGUE. THE BEHAVIOR OF TRICHOMONAS VAGINALIS IN A SEMI-SOLID MEDIUM. M. J. Hogue, University of Pennsylvania Medical School.

When *T. vaginalis* was cultured in a semi-solid medium it often became much elongated. There were two distinct parts to these individuals, a spherical or pyramidal part which was granular and non-motile, and an elongated, non-granular pseudopodium which contained the flagella and undulating membrane. This pseudopodium was in constant motion though the trichomonas did not change its position. After a few minutes this pseudopodium was drawn into the body and the trichomonas swam away. After 48 hours in the semi-solid medium a much longer pseudopodium was often formed. This could not always be withdrawn, though the addition of Ringer's solution sometimes brought these forms back to normal. The density of the medium and the contractile tension of the cytoplasm are important factors in the formation of these long pseudopodia.

HOROWITZ, S., see Park et al. 2

HUMMEL, K. P., see Snell et al.

HUMPHREY, et al. TWO UNUSUAL HAPLOID-DIPLOID MOSAICS OF MIXED AMBLYSTOMA MEXICANUM AND AMBLYSTOMA TIGRINUM ANCESTRY. R. R. Humphrey and G. Fankhauser, University of Buffalo and Princeton University.

Among 3004 offspring of white axolotls mated with animals of axolotl-tigrinum ancestry heterozygous for color, two showed the tigrinum coloration on one side and the white color on the other. Chromosome counts and measurements of nuclei in tail tips demonstrated the dark side to be diploid and the white haploid.

One mosaic, a male, from a white father and heterozygous dark mother, seems adequately explained by assuming that division of the sperm pronucleus initiated cleavage, one of the resulting daughter cells producing the haploid white half of the mosaic, while the other cell, through fusion of its sperm daughter-nucleus with the included egg pronucleus (bearing gene for dark) produced the diploid dark side.

The second mosaic is from a white mother of genotype WW (all offspring females; the normal female is heterogametic, ZW), mated with a male (ZZ) whose only testis is a graft from an axolotl-tigrinum hybrid, his spermatozoa thus carrying the Z chromosome and a gene for either white or dark color. In the mosaic, the dark color of the diploid female side requires participation of a spermatozoon bearing the gene for dark; the testis and white color on the haploid side, however, indicate the origin of that side from a spermatozoon carrying the gene for white. An assumption of dispermy therefore seems essential to explain all the facts of sex and color in this animal. Dispermy could also explain the origin of the first mosaic, although its formation as suggested seems entirely probable.

HUTCHINSON, G. E., see Brooks

ITZKOWITZ, C., see Moody et al.

JAHN. BRIGHTNESS ENHANCEMENT IN FLICKERING LIGHT. Theodore Louis Jahn, University of Iowa.

In recent publications Bartley has pointed out that in human vision as the frequency of a flickering light is lowered to a point below that of fusion, the Talbot effect disappears and the apparent average brightness increases to a maximum which may be twice that of the same light when viewed continuously. It is stated that this brightness enhancement can not be explained by photochemical events in the sense cells, and, therefore, that the explanation must be within the neural part of the optic pathway.

The purpose of the present paper is to point out (a) that a comparable enhancement occurs in photosynthesis where there is no possibility of a neural explanation, (b) that enhancement can be predicted from the steady state equations of the visual photochemical cycle in the light-adapted eye, (c) that the photochemical equation which Bartley considered to be inadequate is apparently that for the dark-adapted eye and should not be used for explaining visual phenomena in the light-adapted eye, (d) that enhancement is a general property of all photochemical cycles, and (e) that the existence of a photochemical explanation does not disprove the neural theory.

The reasons which have been used for eliminating the photochemical theory certainly are not valid, and at present the phenomenon of enhancement apparently can be explained on the basis of either photochemical or neural events.

JAHN, et al. SPECTRAL SENSITIVITY OF THE COMPOUND EYE OF DYTISCUS. Theodore Louis Jahn and Verner John Wulff, University of Iowa.

The electrical response of the compound eye of *Dytiscus* to visible light of various wave lengths was measured. The stimulating light was filtered through Corning glass filters which afforded eight moderately well defined color bands. The intensity was measured with a thermopile and galvanometer and was varied so that the magnitude and wave form of the electrical response could be matched for light of different wave lengths.

The eye of *Dytiscus* is most sensitive to green of a wave length of about 5300 Angstroms. The intensity of blue-violet light (4300 Å) necessary to produce the same response as green light was about ten times that of the green. The intensity of orange red (6400 Å) necessary for the same response was about one hundred times that of green, and the intensity for deep red (7200 Å) was about five thousand times that of the green. In comparison with the human eye it is seen that the spectral sensitivity curve of *Dytiscus* does not slope sharply toward the violet end of the spectrum and that sensitivity in this region is relatively high. This is in agreement with other data obtained by both electrical and tropistic response methods for other insects.

There was no definite detectable difference in the spectral sensitivity curve of *Dytiscus* during the day and night phases of the diurnal rhythm.

JAHN, T. L., see Wulff et al.

JENSEN, D., see Adams et al.

JUHN. THE BEHAVIOUR OF ABNORMAL FEATHER PIGMENTATION IN THE BROWN LEGHORN TOWARD ESTROGENS. Mary Juhn, University of Maryland.

It has been earlier reported that Brown Leghorn males with symptoms of leucosis show a characteristic change to red in the black plumage tracts of the body. In prolonged cases successive feather generations often show a gradual increase in the modified feather region, the extension of the foreign coloring proceeding from the margin toward the shaft, but a fluctuation between the normal and the original pigmentation may also occur.

The effects in the breast tracts of such abnormally pigmented fowl of stilbestrol alone, of theelin alone, and of theelin and thyroxin given in combination, differ according to the phase of pigmentation of the feather or feather segment at the time of the first injection.

In feathers, originally black, theelin and stilbestrol cause typical salmon hen feathering. In feathers laying down red in marginal segments, both stilbestrol and theelin cause the reappearance of black; this is followed by a return to red upon cessation of treatment. In feathers completely red, theelin is apparently ineffective save in increasing barbulation where this has been reduced.

Thyroxin has been shown to cause a return to black in such abnormal feathers. Theelin and thyroxin together lead to a greyish color in feather segments originally red and to salmon in those originally black. The ratio of theelin to thyroxin used covers a narrow range and a modification of procedure may lead to additional reactions.

These experimental results indicate that the abnormal pigment resembles in behaviour the red of the male saddle rather than the female salmon.

KARCZMAR, A. G., see Sandow et al. 1 and 2

KING, R. L., see Beams et al. 1 and 2

LAMAR. EPINEPHRINE EFFECTS ON YOUNG MALE RATS. Jule K. Lamar, The University of Texas School of Medicine.

As part of a general program to investigate factors influencing spermatogenesis, the effect of epinephrine on young male rats was tested. Perry ('41) had found that adrenalin chloride (1:1000 aqueous) Parke-Davis, in daily doses of 0.06 to 0.25 cc. for 14 days caused testicular and sex accessory damage in young male rats, particularly in summer. In the present experiments, of twenty-eight male rats of six litters, eight served as controls and twenty were injected with Epinephrine in Oil 1:500) Squibb, 0.025 to 0.15 cc. daily, intramuscularly, or Epinephrine Hydrochloride Solution (1:1000) Squibb, 0.10 to 0.20 cc. daily subcutaneously. Age at the beginning of injections was 41 to 63 days, and age at autopsy was 62 to 84. Animals were injected 14 to 17 days, and autopsy occurred 4 to 8 days after the last injection. All injections occurred during June, July and August. Five of the injected animals died, four of them after the first or second injection. In the remaining fifteen there was no depression of spermatogenesis or of sperm motility as shown by studies of epididymis and vas deferens content at autopsy, nor was there evidence of testicular damage from histological studies of the testes. Accessory and testis weights were not depressed in the injected animals when put on the basis of percentage body weight, and histology of the seminal vesicles and prostates was normal. Doses which killed some animals left their littermates apparently without harm, and caused no discernible damage to testes or accessories.

LAMAR, et al. SEASON AND HUMAN FERTILITY IN GALVESTON, TEXAS. Jule K. Lamar and Rodney Rodgers,¹ The University of Texas School of Medicine.

Data on the number of births per month and the temperature means of the calculated months of conception for Galveston in the 10-year period 1932-41 show that conceptions were least frequent in July and August, when temperatures were highest (83-84°F.), and most frequent in January, when temperatures were lowest (56°). The percentage increase from lowest to highest conception rates averaged 77%, and was practically identical for Whites and Negroes. The percentage of stillborn (of total births) increased sharply for those conceptions occurring in July, August and September, being up to 134% greater than for conceptions in June. Conceptions in winter had stillbirths at an intermediate

level, 72% above the June low. No correlation occurred between birth rate or stillbirth percentage and the mean relative humidity of the month of conception. Humidity means ranged from 73% in October to 82% in January and February. Conception rate curves show variations from year to year, following in general the inverse curve of temperature at conception time. In only 2 years ('38 and '40) did an increase in conception rate occur before the mean temperature began to fall. Average mean temperature for highest conception rate was 59°, and this generally occurred before or during the lowest mean temperature of winter. In only 2 years ('35 and '41) did the peak of conception rate occur after the lowest mean temperature was past. Work now going on attempts to determine the factors involved.

¹ Upjohn Fellow, 1942-43.

LATIMER. THE WEIGHT OF THE SKELETON IN THE FETAL, NEWBORN AND ADULT CAT. Homer B. Latimer, University of Kansas.

This is a report on the weights of the moist ligamentous skeletons of 229 fetal, 35 newborn and 104 adult cats.

The skeletal weight in the fetal and newborn cats increases as an arithmetical function of the body weight and the same empirical formula fits both the fetal and the newborn skeletal weights. The same weights, plotted on body length, form a curve concave superiorly, or they increase as a function of the cube of the body length. One formula fits both fetal and newborn skeletal weights on body length.

The percentage weight of the fetal skeletons decreases rapidly at first, and then more slowly to 50 gm. of body weight and then averages 12% of the body weight in the older fetal and in the newborn cats. The percentage weights in the adults are 13.84 for the males and 13.38 for the females, or there is no significant difference in the percentage weights.

The adult male skeletons average 378.7 gm. and the females, 325.5 gm., or the absolute weight is significantly greater in the males. The adult male skeleton is more variable in weight and also slightly more variable in its percentage weight.

LAVIN, G. I., see Costello et al.

LEATHEM, J. H., see Babcock et al.

LILLIE, et al. EVIDENCE OF INDUCTION IN FEATHER DEVELOPMENT. Frank R. Lillie and Hsi Wang, Whitman Laboratory of Experimental Zoology, The University of Chicago.

The mechanism of feather development was further investigated by grafting selected parts of papillae and testing their independent power of development separately. A graft of the mid-third of a dorsal half inserted orthotopically in the middle of the ventral surface of whole host-papillae induces a feather twinned face to face with, and equal in all respects to the host partner. Other dorsal

parts, when similarly grafted, show power of induction decreasing toward the equator of the papilla, where it completely ceases. Similar grafts derived from the ventral half have no inducing power but produce only enlarged after-feathers. The independent power of development of the parts used as grafts, when isolated, and their growth rates run parallel to inducing power. Half-vanes are produced from sectors of the papilla containing only a small peripheral portion of the dorsal half immediately above the equator.

When grafts are placed dorsally in the host, thereby dividing equally its dorsal half, equal twins are produced from grafts of ventral origin, and triplets from those of dorsal origin.

These results demonstrate the presence of an inductor in the derma of the dorsal half of the papilla, decreasing in amount from mid-dorsal line to the equator. Its effect is regarded as acceleration of developmental processes in the ectoderm (substratum) and production of gradients of limited range in the collar. Material beyond the range is physiologically isolated; by virtue of autonomous power of development, it produces one or more after-feathers varying in size in proportion to the isolated amount.

LOOSANOFF, et al. GROWTH, INCREASE IN WEIGHT, AND MORTALITY OF MUSSELS, *M. EDULIS* LINN., LIVING AT DIFFERENT DEPTH LEVELS. Victor L. Loosanoff and James B. Engle, U. S. Fish and Wildlife Service, Milford Biological Laboratory.

Large numbers of mussels of uniform size and age were suspended in wire baskets at three levels in Milford Harbor. They were examined at monthly intervals between April, 1942 and May, 1943. The group kept at the mean low water level showed the most rapid growth. The second group kept at the bottom grew somewhat slower. In both groups the growth rate was highest during May and June. In July, August and September the growth rate considerably decreased. During the winter the increment in growth was extremely small but measurable. In the spring a more rapid growth was resumed. The third group kept at +5 feet tidal level showed virtually no increase in length.

The animals of the two lower groups showed an increase in weight throughout the entire period of observation. In general, the increase in weight corresponded to the increase in size. The mussels kept at a +5-foot elevation showed but a slight increase.

The mean low water group showed the lowest mortality rate. Approximately 5% died during the year. The mortality of the bottom group reached 11.5% during the same period. In the majority of cases the death of the animals occurred during the warm season. Approximately 16% of the mussels kept at a +5-foot elevation died between May and November. In December all the remaining animals of this group froze and died.

LOOSANOFF, V. L., see Engle et al.

LUDLAM, H. W., see Romanoff et al.

LYNN. SITUS INVERSUS VISCERUM IN CONJOINED TROUT EMBRYOS. W. Gardner Lynn, The Catholic University of America.

A large series of trout embryos showing various degrees of doubling of the anterior end of the body was dissected. In this series there were 1,087 specimens in which the twin digestive tracts were united at some point behind the stomach so that two stomachs and two livers were present. In 633 (58.2%) of these, both digestive tracts exhibited normal situs; in 80 (7.4%), situs inversus viscerum was found in both members of the pair; in 374 (34.4%) situs inversus viscerum occurred in one member only. Among the latter, the right hand member was reversed in 238 (63.6%) of the cases, the left-hand member in 136 (36.4%). There seems to be no clear correlation between the degree of duplication and the frequency of pairs showing situs inversus in one member, but pairs having both members reversed are much more common in twins in which the duplication extends only to the level of the dorsal fin than in those which are more completely duplicated.

Dissection of 115 pairs of completely divided twins, joined only by their attachment to a common yolk sac, revealed reversal of situs in both members in two (1.7%) cases and reversal in one member in sixteen (14.0%) cases.

Dissection of 510 single embryos showed reversal in twenty-four (4.7%) of the specimens.

It should be noted that all twins in which the duplicity involves only regions anterior to the stomach level, as well as all autosite-parasite pairs have been reserved for later investigation and are not included in the present report.

MacLENNAN. THE GROWTH OF THE CONTRACTILE VACUOLE IN AMOEBA PROTEUS. Ronald F. MacLennan, Oberlin College.

The growth of the contractile vacuole was slowed by the use of a micro-refrigeration chamber which permits continuous observation with an oil-immersion lens. Measurements of diameter were made at 15 second intervals. The pulsatory cycle lasts as long as 20 minutes in normal amoebae at 5°C. thus permitting as many as sixty observations in a single cycle. No effect on the cycle other than slowing was noted. The growth curve is made up of a series of sharply defined steps and is never a smooth curve. All fluid enters the main vacuole by the fusion of accessory vacuoles with the main vacuole, none enters through the membrane of the main vacuole. The presence of a lag period after the systole of the previous pulsatory cycle and the formation of new accessory vacuoles is interpreted as indicating that an important phase of the segregation of fluid is accomplished by the cytoplasm before the formation of any membranes.

MILLER. THE EFFECT OF SODIUM AZIDE AND CYANIDE UPON THE HEART RATE OF ARTEMIA SALINA. Frazie J. Miller, Jr., Atlanta University. (Introduced by Harold E. Finley.)

Zoea stages of *Artemia salina*, the brine shrimp, become sufficiently transparent to permit studies on the frequency of the heart. The average of several counts on the heart rate was made for each individual at a pH of 7.2 and a temperature

of 25°C. before treatment. Then a shrimp was subjected to .001 M concentration of sodium azide and cyanide under the same temperature and pH conditions and new counts were made every 5 minutes for 30 minutes.

A depression of the heart rate occurred in both poisons but to a greater extent with cyanide than with azide. Recovery was possible after a 30-minute exposure in both poisons but more shrimp recovered from azide than from cyanide.

The difference in depression of heart rate by cyanide and azide may be interpreted as meaning that azide is limited to one phase, the activity respiration and that cyanide inhibits both the activity and resting respiration, or that two systems are operative each different in its sensitivity toward these poisons.

These experiments are to be extended to include the effect of lower pH upon rate of penetration and the relationship between molar concentration and degree of inhibition.

MILNE, et al. SELECTION OF COLORED LIGHTS BY NIGHT-FLYING INSECTS. Lorus J. Milne, Johnson Foundation, University of Pennsylvania, and Margery J. Milne, Beaver College, Jenkintown, Pa.

To evaluate the effect of color on the photopositive behavior of night-flying insects under field conditions, a series of experiments was conducted during two summers at the Mountain Lake Biological Laboratory in Virginia. Each night of the experiment special light traps offered a selection of colors to attract insects, and the collections of the traps were studied statistically. Over 18,000 specimens of 660 species were identified by specialists. These data gave information on accuracy of aim in trap approach, on factors affecting the catches of identical traps simultaneously exposed, on the effect of light intensity, on the comparability of catch in different years, on the effect of repeating a given color combination on successive nights, on the attraction of different colors of unequal brilliance, and on the attraction of different colors of equal brightness (photometrically). Different species were found to show different selection behavior, and no color was found to which some species were not attracted in at least equal numbers to those trapped by other colors simultaneously exposed.

MILNE, M. J., see Milne et al.

MOODY, et al. OPALESCENCE OF ANTIGEN AS A VARIABLE IN SEROLOGICAL INVESTIGATIONS WITH THE PHOTONREFLECTOMETER. Paul A. Moody and Charles Itzkowitz, University of Vermont.

The Libby photonreflectometer, used increasingly in studies of serological relationship, adds a new dimension to measurement of the precipitin reaction by making possible the plotting of a complete curve of antigen-antibody reaction, from zero reaction due to antigen excess (at the beginning of the prozone), through reactions in a progressive series of antigen dilutions, to zero reaction arising from insufficient concentration of antigen to produce detectable effect. The reaction is measured in terms of turbidity developed. Hence any extraneous factor affecting the turbidity of the system forms a variable which must be controlled. In relationship studies on *Peromyscus*, initial opalescence of the antigen constitutes such a variable. Degree of opalescence of mouse sera varies from day

to day; seemingly, also, there are racial differences. Serum of high opalescence gives much larger readings in the prozone than does the same serum following reduction of opalescence. With a given antiserum, opalescent heterologous sera sometimes give larger reactions in the prozone than do relatively non-opalescent homologous sera. In one instance there was evidence that the initial peak of a bimodal curve of reaction was an artifact resulting from opalescence of the antigen. A means of measuring relative opalescence with the photorelectrometer was devised. Opalescence can be reduced (1) by starving the animals for 24 hours prior to bleeding, (2) by passing the serum through a Seitz filter, though one filtration is not always sufficient to cause complete clearing. The investigation was aided by a grant from the American Philosophical Society.

MURRAY, et al. BELTED, A NEW RECESSIVE SPOTTING MUTATION IN THE MOUSE.

Joseph M. Murray and George D. Snell, University of Maine and Roscoe B. Jackson Memorial Laboratory.

In 1938 a new recessive spotting mutation, belted (symbol *bt*), arose in the *dba*-strain mice maintained at the University of Maine. Crosses with piebald showed it to be a distinct mutation. In belted animals the area of dorsal white ranges from about 1% to 20% of the total dorsal surface, and the ventral patch through about the same range. Contrary to the case in piebald, the dorsal patch is larger than the ventral in the majority of animals, though the reverse condition is not uncommon. It is usually located posterior to the mid-point of the back. The gene segregates in typical Mendelian ratios. It is linked with *caracul* and *naked*, the order of the genes being *bt* *Ca* *N*, the crossover values 11.5% between *bt* and *Ca* and 2.8% between *Ca* and *N*. This is the third major spotting mutation to be found in the house mouse, the other two being piebald (*s*) and dominant spotting (*W* and its allele *W'*).

NELSEN. SEX DIFFERENTIATION IN THE OPOSSUM, *DIDELPHYS VIRGINIANA*. Olin E. Nelsen, University of Pennsylvania.

The differentiation of sex in the opossum appears to be as follows:

1. Three groups of individuals have been noted during the first day after birth: (a) Those in which the gonad appears entirely indifferent with primary sex cords faintly indicated and a quiescent or slightly active germinal epithelium. Some of the sex cords are still definitely attached to the germinal layer; (b) In others the germinal epithelium is quiescent, and the primary sex cords distinct; (c) In a third group may be placed individuals with definite, tortuous sex cords, a flattened germinal epithelium, and a well-developed tunica albuginea. Of eleven authentic first day animals with good histological fixation, four specimens belong in group a, five in group b, and two in group c.

2. Sex differentiation begins to be evident on days 2 and 3 after birth when the germinal epithelium in the female inaugurates a second phase of proliferation and secondary sex cord formation is initiated.

3. Histologically, sex is clearly indicated on days 5 and 6. Well-formed sex cords, a flattened germinal epithelium, and pronounced tunica albuginea are present in the developing testis, and an active germinal epithelium with forming secondary sex cords are evident in the ovary.

NELSON, et al. EFFECTS OF ADRENAL CORTICAL COMPOUNDS ON LACTATION. Warren O. Nelson, Robert Gaunt and Malvina Schweizer, Department of Anatomy, Wayne University College of Medicine, and Department of Biology, Washington Square College of Arts and Science, New York University.

A further study of the relationship between the lactogenic, adrenotrophic and adrenal cortical hormones in the control of mammary gland activity was made in sixty-nine hypophysectomized, and sixty-one intact guinea pigs, with the following results:

1. Previous findings that lactation could not be induced in hypophysectomized guinea pigs by lactogenic hormone alone, but could be initiated if adrenotrophic hormone or adrenal cortical extract were given with the lactogen were confirmed and extended.

2. Lactation could not be induced in hypophysectomized guinea pigs when desoxycorticosterone acetate was given together with lactogenic hormone.

3. Lactation could be induced in hypophysectomized guinea pigs when 1 mg. of 17-hydroxy-11-dehydrocorticosterone was given daily with lactogenic hormone, although the quantitative response was not as good as with 2 cc. of a commercial adrenal cortical extract (Eschatin).

4. Studies on hypophysectomized animals suggested and those on intact animals confirmed that desoxycorticosterone acetate (3 mg. daily) has a lactation-inhibiting influence. This was probably related to the fact that mammary growth could be induced by this steroid when given in 2 mg. daily doses.

NELSON, et al. FURTHER STUDIES ON THE MAINTENANCE OF LUTEAL FUNCTION IN HYPOPHYSECTOMIZED ANIMALS BY LACTOGENIC HORMONE. Warren O. Nelson and J. Walton Pichette, Department of Anatomy, Wayne University College of Medicine.

It has been shown earlier (Nelson and Pichette, '42, '43) that the pseudopregnancy which can be induced in rats by the injection of estrogens will be interrupted if the hypophysis or ovaries are removed; and that the injection of luteotrophin (lactogenic hormone) in estrogen-treated hypophysectomized animals will effectively substitute for the hypophysis in maintaining pseudopregnancy for periods up to 17 days.

Twenty-two animals received 200 I. U. estrone daily. Each was hypophysectomized 5 to 7 days later. Treatment with lactogen was instituted immediately, estrone being continued. Twelve animals were sacrificed on the 6th to 17th day of such treatment. In each case pseudopregnancy had been maintained as shown by the vaginal smear, histology of uterus, vagina and mammary glands. In ten animals treatment was continued. In each instance the vaginal smear showed a return to estrus on the 19th to 24th day. These animals were killed from 1 to 5 days after their return to estrus, treatment with estrone and lactogen being continued until sacrifice. All of these animals showed luteal involution, estrone effects in vaginae and uteri and some degree of mammary involution.

This study indicates that corpora lutea cannot be maintained indefinitely by luteotrophin administered as lactogenic hormone and affirms the fundamental importance of the ovaries in the stimulation and maintenance of mammary growth.

NEWMAN, et al. ONE-EGG TWINS WITH SPINA BIFIDA AND POLYDACTYLY. H. H. Newman, The University of Chicago, and W. B. Quisenberry, M.D., U. S. Public Health Service.

Recently a pair of Negro female one-egg twins were born to a 24-year-old Negro woman. They were described as monochorionic and monamniotic. Both twins showed similar extensive spina bifida and spastic paralysis of the legs. They were extremely similar in a number of unusual characters. The only noticeable difference consisted of the presence of two well-developed thumbs on the right hand of twin A, the remaining hands and feet of both twins being normal.

Since spina bifida is surely due to developmental arrest, and since the arrest must have preceded twinning, it seems probable that the same arresting factor caused both twinning and spina bifida. A survey of all known relatives of the twins failed to reveal any cases of polydactyly; hence the character can not be inherited as a dominant trait. The possibility of it being inherited as a recessive can not be excluded. The expression of polydactyly in only one of the two genetically identical individuals, and only in one hand of one of them, can not be due to differences between them in genetic milieu or to differences in external environment. It is believed to be due to a right-left metabolic differential in the embryo prior to twinning and to the same sort of differential in twin A. The possibilities that this case of polydactyly was due either to a new mutation or to a somatic modification are also examined.

NOLAND. A NEW METHOD FOR LETHAL TEMPERATURE DETERMINATIONS; STUDIES ON PERIPLANETA AMERICANA. Jerre L. Noland, Purdue University. (Introduced by Wm. A. Hiestand.)

A suitable apparatus has been constructed for determining the lethal temperature of insects by which the environmental temperature can be increased at a constant rate in preference to prolonged exposure to a constant temperature. A gas mixer, a humidifier, or a desiccator may be used to condition the air which is preheated to the water-bath temperature by passage through coiled glass tubing, and which is finally circulated through the animal chamber at a measured rate.

The lethal point is provisionally expressed as the temperature at which death occurs in a homogeneous group of insects after exposure to a uniform temperature rise of 1° every 5 minutes. A laboratory colony of roaches was found to yield reproducible results which indicate that the lethal temperature as defined above is a biological constant. Unfavorable conditions such as moulting, physical injury, and poisons all lower the lethal temperature. Low humidity decreases the lethal temperature of newly hatched nymphs, presumably because of excessive water loss which may amount to one-third the body weight. After the third moult water loss is still evident, but the lethal temperature in dry air remains unchanged. Very small nymphs have considerably lower L. T. than adult roaches. This was found to be a hyperbolic function of the weight (in mg.) which could be expressed by the equation:

$$L.T. = 51.54 - \frac{0.6}{\log wt. - 0.306}$$

It is postulated that this equation (with different constants) might be valid for other species of insects.

OLIPHANT. THE EFFECT OF A NUMBER OF CHEMICALS ON GROWTH OF *NEUROSPORA CRASSA*. Joseph F. Oliphant, Stanford University.

Wild type *Neurospora crassa* grows very well on a "basic medium" described by Beadle and Tatum having the following composition: NH_4 tartrate, 5 g.; NH_4NO_3 , 1 g.; mg. $\text{SO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; NaCl, 0.1 g.; CaCl_2 , 0.1 g.; sucrose, 15 g.; biotin (as SMA concentrate) 4 gammas; and the "trace elements" B, 0.01 mg.; Cu, 0.04 mg.; Fe, 0.20 mg.; Mn, 0.02 mg.; Mo, 0.02 mg.; Zn, 2.0 mg.; and H_2O 1 liter.

Growth as measured by dry weight of mycelia produced in a given time is inhibited in the absence of the "trace elements". Growth increases as the concentration of "trace elements" is increased up to 3x. From 3x to 50x growth remains relatively constant. In higher concentrations there is an inhibition. Fe influences growth more profoundly than the other "trace elements".

There is no growth if biotin is omitted nor is there growth in the absence of K, PO_4 , Mg, or SO_4 . Growth is inhibited in the absence of NaCl and CaCl_2 . Nitrogen and carbon sources may be varied widely.

The tolerance of *Neurospora* for fifteen salts, all chlorides, was ascertained. Some growth was obtained in 2N, K and Na and in N solutions of several salts. Growth equal to controls was obtained in N/20, K, Na, NH_4 , Mg, Mn, Sr. Heavy metals were more toxic in the order $\text{Hg} > \text{Fe} > \text{Al} > \text{Ba}$.

OLIVER. A CASE OF CHANGE IN DOMINANCE OF A DOMINANT MUTANT IN *DROSOPHILA MELANOGASTER*. C. P. Oliver, University of Minnesota, Minneapolis.

The mutant punch eye-color is dominant to its normal allele but behaves as a recessive when heterozygous with a translocation (T24A34). Punch is associated with an interchange of segments of the right arm of chromosome 2 and the left arm of chromosome 3 and is homozygous lethal. Punch has been reversed to normal by irradiation both with respect to the eye pigment and the lethality. Translocation-24A34, received from Texas University, involves chromosomes 2 and 4. Flies heterozygous for the translocation have the normal eye pigment. In flies heterozygous for punch and -T24A34, the punch is completely suppressed and the eyes have normal pigment. Thus in association with the translocation, punch loses its dominance and behaves as a recessive character. Flies punch/-T24A34 produce only normal offspring due to the lethal associated with Pu; in the heterozygous culture, punch behaves as a recessive lethal. Flies Pu/T24A34 mated to normal flies produce normal (heterozygous T24A34) and punch offspring. Crossovers show that the suppressor or modifier of punch is associated with the right arm of chromosome 2, the arm which is involved in punch and in T24A34. This is a case of a mutant which is dominant in comparison with the regular "normal" culture but which in association with genes in another culture becomes recessive in expression and therefore has evolutionary significance. Whether the change in dominance is due to modifiers or to another allele of Pu which also produces normal pigment cannot yet be stated.

PAINTER. THE EFFECTS OF ALKALI AND UREA ON DIFFERENT TYPES OF CHROMOSOMES. T. S. Painter, University of Texas.

Mitotic and meiotic chromosomes of the plant *Rhoeo* and salivary chromosomes of *D. melanogaster* have been treated with a "50%" (Kodani) solution of NaOH and urea. All the chromatin in resting nuclei (haploid or diploid) may form a single typical lampbrush chromosome; first meiotic and salivary chromosomes produce similar images, the series of changes being the same in all three cases. (1) Immediately after exposure formed structures (granules and bands) disappear and the chromatin becomes dispersed. This possibly is due to the breaking of the salt linkage between thymonucleic acid and the protein backbone of chromosomes. Simultaneously (ca. 1 to 5 s.) there is a violent shrinkage of whole nuclei or chromosomes which compresses the freed chromatin into folds of the nuclear wall, or to the center of chromosomes as disjointed or continuous rods. (2) In 5 to 10 seconds nuclei or whole chromosomes begin to swell very rapidly by the formation of clear Feulgen negative vesicles, small within and largest about the periphery of nuclei or chromosomes. This is due, presumably, to the hydration and hydrolysis of the protein backbone. The vesicles seem to squeeze the plastic chromatin between their interfaces. (3) The vesicles rupture and/or their contents merge with the cytoplasm leaving innumerable hairs of chromatin radiating out from a core of this material. This gives the lampbrush image. It is concluded that this lampbrush form taken by the chromatin of whole haploid or diploid resting nuclei, one or several fused meiotic and salivary chromosomes, is due first to a detachment of the chromatin from the protein substrat and subsequently its moulding by forces incident to the swelling and dissolution of the protein backbone of chromosomes. In view of these findings we are justified in regarding with great reserve any theory of salivary chromosome structure based on dissolution images.

PARK. INTER-SPECIES COMPETITION WITHIN POPULATIONS OF THE GENUS *TRIBOLIUM*. Thomas Park, Hull Zoölogical Laboratory, The University of Chicago.

This is a long-term project on the competitive effects existing within populations of the flour beetles, *Tribolium confusum* and *Tribolium castaneum*. The growth and maintenance of these two forms is first observed when they are cultured as single species populations. This series constitutes the control in the sense that there is no inter-species competition operating within these cultures. The experimentals, or mixed-species populations, fall into three series: those designed to give no initial numerical advantage to either form; those that favor *Tribolium confusum*, and those that favor *Tribolium castaneum*. The entire study is run under four different flour-volume relationships with the environmental conditions as favorable and as controlled as possible. Certain preliminary findings can be reported at this time: (1) *Tribolium castaneum* produces larger total populations than *Tribolium confusum* although the latter cultures have a higher percentage of imaginal forms; (2) Cycles of growth are evident for both species; (3) Inter-species competition reduces total population size, and (4) Of the two forms, *Tribolium castaneum* appears to be the more successful competitor in the population (statistical) sense.

PARK, et al. FURTHER ANALYSIS OF FECUNDITY IN THE FLOUR BEETLES, *TRIBOLIUM CONFUSUM* DUVAL AND *TRIBOLIUM CASTANEUM* HERBST. Thomas Park and Marion Biggs Davis. Hull Zoölogical Laboratory, The University of Chicago.

(1) Records of the oviposition of *Tribolium confusum* and *Tribolium castaneum* over a 60-day period of observation are analyzed. It is shown that the latter species has a significantly higher fecundity rate by about 6% than the former species. The variability is greater for *Tribolium castaneum* also. (2) On the basis of selection of high and low strains of beetles it was possible to develop a stock of *Tribolium castaneum* with a 15% greater fecundity rate than the parental generation. It was not possible to do this for *Tribolium confusum*. Some suggestions that may account for this failure are presented. (3) Analysis of variance of the total data shows conclusively that there is more variability in respect of fecundity between pairs of beetles than within pairs.

PARK, et al. "EBONY": A NEW MUTATION IN THE FLOUR BEETLE, *TRIBOLIUM CONFUSUM* DUVAL. Thomas Park, Marion Biggs Davis and Shirley Horowitz, Hull Zoölogical Laboratory, The University of Chicago.

A mutation has appeared in laboratory cultures of the flour beetle, *Tribolium confusum*. This mutant is called "ebony" because it changes the beetles' normal red-brown color to a shade that is nearly black. Breeding experiments indicate that the ebony effect is due to a single recessive gene. This is the second mutation to be described for the genus; the other is the gene "pearl" which when homozygous removes pigment from the central ommatidia of the eyes of *Tribolium castaneum*. There are suggestions that the ebony beetles are less fecund than the normal type and take longer to metamorphose. This is being studied. The ebony gene is of particular value in that it provides a readily distinguishable character which can be used as a "marker" in population research.

PARKER. COLOR CHANGES IN THE AMERICAN EEL *ANGUILLA ROSTRATA*. G. H. Parker, The Biological Laboratories, Harvard University.

Like European eels (Waring, Vilter, Waring and Landgrebe) American eels blanch and darken under appropriate surroundings and with considerable quickness. On a white background the American eel becomes pale in about 3 hours and on a black one it becomes dark in about an hour (22°C.). These intervals are shorter than those given for European eels which seem to have been studied at lower temperatures.

Only one set of records are at variance with those already given. Neill declares that the color changes of European eels require some 20 days instead of a few hours. When Neill's records are inspected, however, it is evident that he has included in his tabulations two very different operations, the so-called physiological and the morphological changes. As his plottings show the physiological change is a nearly complete color change and is accomplished in a few hours. It is what has usually been recorded by authors as the full color change. It is followed according to Neill by a very gradual change which stretches over almost the whole of the 20-day period. This is the morphological change. As is well

known the physiological change is due to pigment migration within melanophores; the morphological one to an increase or decrease in the number of melanophores. These distinctions seem to have escaped Neill's attention. Hence his misleading statement concerning the times for color changes in the eel.

PEARSE. CHELONETHIDA FROM THE DUKE FOREST. A. S. Pearse, Duke University.

In routine collections from the litter and soil in the Duke Forest, at all seasons, four species of chelonethids were found in the numbers indicated: An unidentified cheliferid, 6; *Microbisium brunneum* (Hagen), 122; *Chthonius ischnocheles* (Hermann), 121; and *Apochthonius crosbyi* Chamberlin, 140. Sixty per cent came from two stations in a pine forest on sandy loam and 40% from two stations in an oak forest on clay. In the former there were 156,816–214,616 individuals per acre and in the latter 169,821–196,020. In litter 89.4% occurred; in the upper 2 inches of soil, 5.5%; and at depths of 3–5 inches, 5.1% Chelonethids increased in number after rainy periods.

PEASE. SURFACE MOVEMENTS DURING THE CLEAVAGE OF ECHINODERM EGGS. Daniel C. Pease, Stanford University, Hopkins Marine Station, Pacific Grove, California.

Katsuma Dan and his co-workers (see particularly *Cytologia*, 8: 512, '38) have measured the linear surface movements of naked sea urchin eggs during cleavage by following the spreading of adhering kaolin particles. But the changes of surface geometry which result from cleavage are such that their linear measurements give no a priori information about the changes in surface areas, and hence any possible cortical dilutions. The geometrical problem is such that their data are not sufficiently accurate to convert directly into terms of area. It was possible, however, to make arbitrary assumptions of surface area changes, mathematically calculate the implications of the assumptions in terms of linear arc extension, and compare these values with the experimental data. By trial and error comparisons it was found that, after the first cleavage and during interkinesis, the original egg cortex comes to be uniformly expanded by approximately 15%. About 11% of the total surface is covered by cortex formed de novo which Dan et al. found to be deposited in the cleavage region after the furrow incutting is completed. If these basic assumptions are appreciably changed, there is no longer any agreement with the experimental data. During furrow intrusion, however, the egg cortex is differentially expanded. It has not been possible to obtain a precise mathematical expression for this process, but the maximal expansion greatly exceeds 26% near the spindle poles, and is rather less than 26% in the furrow region.

PEASE. THE PHYSICAL STRUCTURE OF NEMATODE EGG CYTOPLASM. Daniel C. Pease, Stanford University, Hopkins Marine Station, Pacific Grove, California.

A strain of free-living soil nematodes of the *Rhabdites* type was isolated and bred in pure culture. The strain was selected to have eggs relatively free of inclusions, and a maternal body wall and uterus which were almost transparent. Eggs in utero were studied in living worms with the highest oil immersion magnifications, and the observations checked on eggs just after isolation (and also on different species from mixed cultures).

Brownian movement of inclusions was found to be definitely limited, leading to the belief that a gel network permeated the cytoplasm, and particle movement was restricted to the sol interstices. Most of the cell inclusions were free in the sol phase. The contrast between normal eggs and recently centrifuged eggs was striking. Moderate centrifugal forces solated the gel network, and Brownian movement was unrestricted until the gel slowly reformed, the process taking some minutes to be completed. Rhumbler and Spek have observed streaming movements and the formation of hyaline regions, particularly in association with cleavage. The "streaming" is almost certainly due to local contractions and expansions of the gel network (possibly associated with gel-sol transformations). The normally occurring hyaline caps, most common near the spindle poles, result from syneresis (phase separation resulting from the contraction of the gel). Inclusions left in the hyaline regions showed unrestricted Brownian movement. Unlike sea urchin eggs, amoebae, and many other well known cells, there was no evidence of an appreciably thick gelled cortex. Brownian movement extended all the way to the delimiting interface.

PIATT. EXPERIMENTS ON THE EMBRYOLOGICAL ORIGIN OF THE MESENCEPHALIC V ROOT CELLS IN AMBLYSTOMA. Jean Piatt, University of Pennsylvania.

Extirpation of neural crest material was made on embryos of *Amblystoma punctatum* to determine whether the mesencephalic V root cells arise from this source. Unilateral and bilateral elimination of crest cells throughout the midbrain region was accomplished by either scraping (stage 25) or neural fold removal (stage 16). Sixty-three cases survived.

No significant discrepancy was found to exist between comparably aged normal and experimental larvae with respect to the mesencephalic V root cell count. Range of advanced normal larvae was 140-208 cells; advanced experimental larvae was 129-249 cells. Chondrocranial and hyobranchial cartilages were approximately normal in all advanced experimental larvae. The hyoid apparatus, Meckel's cartilage, palatoquadrate and anterior trabecular bars were absent or very deficient in young larvae. These young larvae also showed the presence of mesencephalic V root cells even though their cartilage deficiencies indicate the effective removal of neural crest. Non-deficiency of cartilages in older larvae may be due to post-embryonic restitution.

Posterior tectal cells frequently defy positive determination as mesencephalic V root cells and often intergrade. For this, and other technical reasons, the results given here should not be too strongly construed as evidence against the thesis of neural crest origin for mesencephalic V root cells in this form. They suggest, however, that mere resemblance of these cells to the Rohon-Beard cells of the cord is not sufficient reason to allocate to them a similar origin from neural crest material.

PIKE. THE THERMODYNAMIC IRREVERSIBILITY OF PROCESSES IN LIVING ORGANISMS.
F. H. Pike, Columbia University.

Confusion has arisen concerning certain processes which have been called reversible, in living organisms. For four decades, biologists have searched for so-called reversible processes comparable to the so-called reversible processes of general chemistry. The object was to disprove the existence of supposed vital characteristics of living organisms. A number of these processes have been found, although there has been scepticism concerning their actual reversibility under biological conditions. In these four decades, we have come to recognize more clearly that, in inorganic nature, processes which are not strictly mechanical are irreversible, thermodynamically.

Two costs must be reckoned in non-mechanical processes, — that in free energy, and that in entropy. Unless both can be kept at zero when the operation is put through in the reverse direction, the process is irreversible thermodynamically. "If an irreversible process can occur, it will occur." (Houstoun). Unless there is some mysterious mechanism in living organisms to forbid irreversible processes, they will occur. Experimentally, we have found many such, but we have never found one which is reversible thermodynamically. To show the existence of reversible processes in living organisms would be equivalent to a demonstration of vitalism.

These considerations bear on certain hypotheses of heredity, the transmissibility of the effects of the environment, and the degeneration of specialized tissues in changed environments, some of which hypotheses postulate thermodynamically reversible reactions, or their equivalent in thermodynamic efficiency.

PIKE. ENTROPY AND THE DEGENERATION OF PHOTORECEPTORS IN PERPETUAL DARKNESS. F. H. Pike, Columbia University.

It has been asserted that degeneration of eyes of cave forms is due (1) solely to chromosomal changes, and (2) to inheritance of the effects of the environment. Since photoreceptors appear only in species which have some time lived in the light, it seems permissible to assume some causal relationship between light and photoreceptors until demonstration of their complete independence.

The chromosomal hypothesis of degeneration of the eyes of cave forms postulates that the process of heredity will go on for generation after generation, with no dissipation of free energy or gain in entropy, and, hence, be a truly thermodynamically reversible process, or a process of equivalent thermodynamic efficiency, such as is found nowhere else in nature.

Since darkness is merely the absence of light, and cannot be a form of energy, it can have no positive effect, and can produce nothing. Since it has no positive effect, the effect of darkness could not be transmitted hereditarily.

The observed result of living in perpetual darkness is what we would expect when organisms experience a failure of the driving force concerned in the development of photoreceptors.

When the phenomena of recession and degeneration, of use and disuse, in ontogeny and in phylogeny, are regarded from the dynamical point of view, there appears to be some fundamental relationship to their irreversibility, in the thermodynamic sense of irreversibility.

PLAGGE. EFFECTS OF HYPOTONIC SOLUTIONS UPON THE LIVING THYROID GLAND.
James C. Plagge, University of Maryland School of Medicine.

The thyroid gland of the living salamander, *Triturus torosus*, exhibits striking differences in its cytology when it is dissected from its fascial surroundings while in 100% and hypotonic Ringer solutions. Marked intracellular disturbances, consisting of an increase in permeability, swelling, and excessive Brownian movement of granules in the cytoplasm, result if the operation to expose the thyroid gland is performed while it is in 30% Ringer solution, but not while in a 100% Ringer medium. If 100% Ringer is used during the operation, and is then replaced by the hypotonic solution, the cells remain essentially unaffected. Both factors—the hypotonic medium and the mechanical disturbances of the operation—must operate simultaneously to induce the changes. The observations suggest that the hypotonic medium increases the sensitivity of the thyroid cell to mechanical injury.

Three to 6 hours after the cellular swelling and Brownian movement have been induced by the operation in hypotonic Ringer, the cells return spontaneously to their standard appearance, that is, to the condition associated with 100% Ringer.

One thyroid in each of forty-five salamanders was studied.

QUISENBERRY, W. B., see Newman et al.

REED. UNILATERAL MEMBRANE FORMATION IN THE SEA URCHIN EGG TREATED WITH ULTRA VIOLET LIGHT. Emerson A. Reed, Stanford University. (Introduced by Arthur C. Giese.)

Eggs of *Strongylocentrotus purpuratus* were irradiated on one side with ultra violet light of wavelengths 2537 and 2483 Å. Upon subsequent fertilization they produced asymmetrical membranes, with the side which was irradiated being suppressed. The shaded side produced a membrane thicker than normal. Under this, the cytoplasm was flattened. In a typical case it became almost a perfect hemisphere. Eggs which were spun around during the exposure so as to irradiate all sides of them formed only very small blisters scattered over all of their surfaces. These eggs remained essentially spherical.

The flattening of the cytoplasm is interpreted as being due to pressure developed within the unilateral perivitelline space since, when such irradiated and fertilized eggs were immediately placed into isotonic urea solution, the membranes broke down, and at the same time the cytoplasm rounded up. Eggs which had been treated with urea solution prior to irradiation and fertilization failed to form membranes, and also failed to flatten.

The perivitelline space of both normal and irradiated eggs has been observed to be traversed by numerous, fine, radial striations. Between these, occasional fragments can be seen to be in Brownian movement. When these contact the striations, they adhere to them. Such striations might be the walls of closely appressed, turgid vesicles derived from certain surface granules upon fertilization.

The effect of the radiant energy on the membrane formation might be the result of its action on these granules.

REESE. THE ANATOMY OF THE POISON GLANDS IN THE BLACK WIDOW SPIDER, *LATRODECTUS MACTANS*. A. M. Reese, West Virginia University.

The two glands are located in the cephalothorax and open at the bases of the chelicerae. They are white, slightly curved sacs varying in length from 1.5 mm. to 2 mm. and about 1 mm. or less in diameter.

Three chief layers in the wall of the gland may be made out. (1) the external, muscular, (2) the internal secreting, (3) the connective tissue, basement membrane between the other two. The muscular layer consists of more or less rectangular fibers that apparently extend from end to end of the gland.

The structure of the secreting layer is very variable in appearance and difficult to describe. It consists of a series of fibro-granular elevations or ridges to which are attached the oval or pear-shaped secreting cells, which are sometimes clear and sometimes granular.

The basement membrane is always distinct varying considerably in thickness in different sections. It seems to be a non-cellular sheet of connective tissue.

REINHARD. EPICARIDEAN PARASITES OF *PAGURUS LONGICARPUS* AT WOODS HOLE. Edward G. Reinhard, Catholic University of America.

In August and September, 1943, 8748 specimens of *Pagurus longicarpus* Say were gathered from shallow water in Great Harbor, Woods Hole, Mass. Of these, 110 or 1.25% were parasitized by the bopyrid *Stegophryxus hyptius* Thompson, which attaches externally to the crab's pleon. All sizes of crabs were infested, the frequency of infestation for each size class being proportional to the frequency of the class in the general population. Distributed by sexes, the host count showed fifty-one infested males and fifty-seven infested females (two parasitized crabs were lost before the sexes were determined). It is interesting that the percentage of total infestation shows no deviation from that reported by Thompson ('01) who found about 1.5% of *P. longicarpus* parasitized by this bopyrid in Great Harbor in 1898.

Another parasitic isopod was discovered inhabiting the body cavity of the same host in the same locality. Infestation by this parasite was slightly less than 1%. This animal represents a new species of an undescribed genus allied to *Entoniscus* of the *Entoniscidae*. It will be formally described in a later publication. No members of this family have previously been reported from hermit crabs. It differs from all known *entoniscids* in communicating with the exterior not by way of the host's branchial chamber but through an opening in the head or eyestalk of the crab.

RICHARDS, et al. PLASTIC COVER GLASS SUBSTITUTES IN MICROSCOPY. Oscar W. Richards, John H. Small and P. Wardham Collyer, Research Department, Spencer Lens Co., Buffalo, N. Y.

To determine whether plastic substitutes for cover glasses on microscope slides affect the performance of the microscope their optical constants were determined. The plastic covers and glass cover glasses were mounted also on silvered slides to form Star Test Plates. These were studied by competent observers. The thicker plastic cover glasses, now on the market, are satisfactory when mounted to give

a plane surface. Optically inhomogeneous materials, of irregular thickness, those that curl or do not have plane surfaces, adversely affect the performance of the microscope and should not be used. Since the substitutes are softer than glass they must be protected from abrasion. It is recommended that thicknesses of 0.18 mm., and none outside of a range of 0.12 to 0.20 mm. be used. For critical observation with unimmersed objectives of high aperture, the correction collar should be set for best results to the position corresponding to the actual thickness of the cover slip, just as would be done with glass cover glasses.

RISLEY, P. L., see Hartley

RODGERS, R., see Lamar et al.

ROGERS, H. M., see Hiestand et al. 3

ROMANOFF, et al. THE EFFECT OF ULTRAVIOLET RADIATION ON THE HATCHABILITY OF AVIAN EGGS. Alexis L. Romanoff and H. W. Ludlam, Cornell University.

Over 600 White Leghorn (*Gallus domesticus*) fertile eggs, in lots of forty-eight, were exposed for periods varying from 2 to 4 hours daily at different stages of embryonic development to an unfiltered ultraviolet radiation. The output of the quartz mercury lamp (Cooper Hewitt) in use was approximately 8.5×10^6 ergs/mm.²/min. at a distance of 23 inches from the eggs, as determined calorimetrically (Romanoff, J. Cell. Comp. Physiol., 21: 123-127, '43). This total energy undoubtedly included, besides the ultraviolet, also some near-visible and infra-red radiation. During the exposures, directly in the incubator, special care was taken to remove the excess of heat and to prevent the accumulation of ozone generated by the lamp.

The results of these studies indicated that the number of chicks hatched from the lots of exposed eggs was nearly the same as that from the control groups. On the other hand, it was evident that in all cases the ultraviolet radiation accelerated the embryonic development, resulting in hatches from 12 to 24 hours earlier.

SANDOW, et al. THE EFFECT OF POSITION OF SHOCK ON THE MECHANICAL EVENTS OF THE LATENT PERIOD OF MUSCULAR CONTRACTION.¹ Alexander Sandow and A. G. Karczmar, Washington Square College of Arts and Science.

The latency mechanical events (latency-relaxation) of the isometric contractions of the frog sartorius have been determined by the piezo-electric cathode-ray oscillographic technique as a function of the position of the shock along the length of the muscle. In all positions the shock-electrode separation was 7.5 mm. Normal and curarized muscles have been used, but in all cases the shock strength was so chosen as to evoke direct excitation of the muscle fibers to maximal tension output. L_R , the time interval between the instants of stimulation and of the beginning of the latency-relaxation, is a fairly linear function of the distance, D , of the cathode from the end of the muscle connected to the stylus of the crystal pickup, irrespective of the polarization of the shock. Extrapolation to

$D = 0$ for a muscle under 3 gm. initial tension gives a limiting minimum of L_R (at 22°C.) of about 0.85 ms. R , the magnitude of the relaxation, decreases, apparently linearly, with D . These results indicate that the latency-relaxation is propagated along the muscle-length as an elastic wave with constantly diminishing amplitude, but with constant velocity. The velocity of the wave in a muscle under an initial tension of 3 gm. is about 4.0 cm./ms., and it is increased when the initial tension is increased. The decrease in R with D is attributed to the viscosity of the muscle medium. The propagation of the positive tension wave seems to follow a somewhat different law which at present is yet unclear.

¹ Aided in part by a grant from the Penrose Fund of the American Philosophical Society.

SANDOW, et al. THE EFFECT OF HIGH TEMPERATURES ON THE INACTIVATION OF MUSCULAR LATENCY-RELAXATION.¹ Alexander Sandow and A. G. Karczmar, Washington Square College of Arts and Science.

The effect of temperatures through the range 30–42°C. on the latency-relaxation of the frog sartorius has been determined in experiments in which the temperature of the muscle has been: (1) gradually raised through the entire range; (2) quickly raised to a given value and then kept there for several hours; and (3) kept at a given value for a certain time and then restored to a lower temperature (25°) in order to test for the reactivation of the latency events. Wherever the technical procedures permit comparison, the results of these experiments are in general agreement with previously announced temperature studies (Fed. Proc., vol. 2, no. 1, p. 43, '43). But the present experiments indicate: (1) that relatively sharp reductions of the magnitude of the relaxation, R , occur around 36–38°; (2) that the possibility of reactivation of R is relatively sharply reduced in muscles that have been exposed to temperatures of about 36–38°; and (3) that complete elimination of R , and of the possibility of its reactivation, result from exposure to temperatures above 40°.

These results may be correlated with the distinction between thermal contraction and thermal rigor of live frog muscle and with Mirsky's distinction between primary and secondary heat denaturation of extracted frog myosin. It is therefore concluded that the latency-relaxation of the stimulated muscle is a function of the contractile myosin.

¹ Aided in part by a grant from the Penrose Fund of the American Philosophical Society.

SAYLES. IMPLANTATION OF A POSTERIOR PIECE OF NERVE CORD IN THE TERMINAL WOUND OF A *CLYMENELLA* REGENERATING ANTERIORLY. Leonard P. Sayles, College of the City of New York.

Implants were pieces of nerve cord and neighboring tissues taken from the posterior region of donors. The first five segments were removed from a worm (158 cases). Then the implant was inserted, posterior end first, through a small dorso-lateral opening in the sixth segment and pushed forward until its posterior end protruded from the open, terminal wound of the host. A small part of the

anterior end of the implant was left exposed at the insertion point. The posterior end of the implant was thus incorporated in any tissue which developed in anterior regeneration.

Of fifty-one survivors not one produced new tissue at the anterior end of the implant. Two hosts produced no terminal new. Twenty-six formed heads varying in organization from complete ones of excellent shape to some with head features barely discernible. Thirteen specimens produced irregular masses of new material showing no head or tail features. Two formed heads with a cone of new material at the posterior end of the implant. Nine developed buds consisting of a head with a tail on the side near the implant. Three other buds consisted of a tail at the implant and another part of doubtful organization. Two produced only a tail bud.

Under these circumstances, therefore, the host's head-forming factor usually prevents tail formation. In about 25% of the cases, however, the implant was able to organize a tail bud. Nearly half of the last-named group showed partial or complete inhibition of head formation.

SCHARRER. THE INFLUENCE OF THE CORPORA ALLATA ON EGG DEVELOPMENT IN AN ORTHOPTERAN (*LEUCOPHAEA MADERAE*). Berta Scharrer, Department of Anatomy, Western Reserve University.

From adult females of *Leucophaea maderae*, a viviparous roach, the corpora allata were removed 1 to 5 days after emergence. The animals were killed and fixed for histological study at intervals from 41 to 65 days after operation. In none of the twenty-nine allatectomized specimens had the eggs developed beyond the stage typical of the freshly emerged imago (length 1 to 1.2 mm.), whereas in normal control animals after the same periods of time embryonic development was well under way. In another control series of normal females starved after emergence and surviving up to about 40 days no egg maturation was found to have taken place. The effect of allatectomy on egg development cannot, however, be attributed to starvation possibly caused by injury to the esophagus at operation. All of the twenty-nine specimens described here were observed to feed readily before as well as after allatectomy, and at the time of fixation (which was beyond the life expectancy of starved animals) they had the appearance of normal, well nourished adults. The results reported are in agreement with the findings in Hemiptera (Wigglesworth) and Diptera (Thomsen, Vogt, Day). The establishment of the role of the corpora allata in egg development in a representative of the Orthoptera is of interest as it supports the findings of Pfeiffer in *Melanoplus* against the negative result of Pflugfelder in *Dixippus*.

SCHNEIDERHAN, M. A., see Hall, E. K., et al.

SCHWEIZER, M., see Nelson et al. 1

SCOTT. AGE AS A FACTOR AFFECTING ALLELOMIMETIC SOCIAL DOMINANCE IN A SMALL FLOCK OF SHEEP. J. P. Scott, Wabash College.

Allelomimetic dominance (social control achieved through mutually imitative behavior) was tested in a flock of ten sheep consisting of two ewes and their

descendents born during a period of 3 successive years, all raised on a 6-acre lot with a minimum of interference. The test consisted of driving pairs of sheep in all possible combinations through an unfamiliar opening into a dark shed, the sheep obviously fearing both driver and opening. The seven offspring of the three mature ewes present were all led by their respective mothers. Of the twenty-six other combinations involving a younger and older sheep the elder led in twenty cases. Of the six exceptions three involved one old ewe partially blinded by wool over the eyes and extremely reluctant to enter the opening, and three included cases in which a lamb was called ahead by its mother or a mother called back by a lamb. It is concluded that age is an important factor affecting allelomimetic dominance in this flock, and that it probably becomes so because of an extension of the tendency of a lamb to follow its mother.

SHETTLES. THE EFFECT OF LOW OXYGEN TENSION UPON REPRODUCTION IN THE MALE GUINEA PIG. Landrum B. Shettles, Department of Obstetrics, Johns Hopkins University and Hospital, Baltimore, Md.¹

It is well established that fowl, rabbits, cats, rams, pigs, horses, cattle, and men when transferred from sea-level to various altitudes in the Andes may become sterile, eventually becoming acclimatized, and then show no impairment of fertility. In so far as is known the exact cause of this effect has not been solved. Low oxygen tension has been suspected, and in order to evaluate this contention, male guinea pigs of known fertility were kept in an atmosphere containing 10 volumes per cent oxygen, equivalent to 19,000 feet altitude, for as long as 89 days. The following observations were made: Mating behavior was completely lacking when females in estrus were placed near these males; ejaculation produced by electrical means at weekly intervals showed a marked decrease in volume and coagulation of the semen, and in number and grade of motility of the spermatozoa. After about 60 days, numerous pin-headed spermatozoa and many white blood cells were observed in the semen. Moderate weight loss occurred, but control animals kept in the chamber containing 20 volumes per cent oxygen, sea-level equivalent, and restricted in their food intake so that an equal or greater weight loss was produced, showed no departure from normal. There was no evidence of acclimatization in so far as this study was concerned. Return of animals to atmospheric oxygen showed complete reversal of changes produced.

¹ Fellow, National Committee on Maternal Health.

SIZER. EFFECTS OF TEMPERATURE ON THE CATALASE-HYDROGEN PEROXIDE SYSTEM. Irwin W. Sizer, Massachusetts Institute of Technology.

Crystalline catalase, recrystallized once, was prepared from beef liver according to the method of Sumner and Dounce. The breakdown of 0.01N H₂O₂ at pH 6.9 catalyzed by catalase was followed by measuring oxygen evolution at temperatures from 0 to 68°C., using the Warburg-Barcroft apparatus. The reaction follows the zero order equation for a brief period at each temperature up to 45° and increases with temperature in accordance with the Arrhenius equation with an activation energy of 4,200 cal. per mole. Above 45° the reaction proceeds more slowly than the expected rate due to the inactivation of the catalase by heat. This inactivation

was studied both in the presence and in the absence of substrate and was found to follow the monomolecular equation. When inactivation of catalase is studied in the presence of H_2O_2 the activation energy for catalase denaturation must be corrected by adding to the measured value 4,200 cal., the activation energy of the enzyme-catalyzed reaction. When studied in the absence of H_2O_2 no such correction is necessary. In the latter method the catalase at pH 6.9 was heated for 5 minutes at the inactivating temperature, then rapidly cooled, and the residual activity determined by adding H_2O_2 at 35°C . With either method the activation energy for catalase inactivation is about 57,000 cal., but there is evidence that this value increases to about 110,000 cal. above 63°C . These high values are similar to those for protein denaturation by heat and indicate that the loss in activity by catalase at high temperatures is due to protein denaturation.

SMALL, J. H., see Richards et al.

SNELL, et al. A TECHNIQUE OF ARTIFICIAL INSEMINATION IN MICE. George D. Snell, Katharine P. Hummel and Walter H. Abelmann, Roscoe B. Jackson Memorial Laboratory. . .

A colony of mice was maintained in a darkroom with lights turned on from 4 P.M. to 4 A.M. Under these conditions there is a sharp peak in estrus onset at 11 A. M. Following the weaning of each female's litter, vaginal smears were taken every morning until estrus was indicated. Between 11 A. M. and noon females presumably in heat were inseminated artificially. An especially designed speculum was inserted into the vagina of the female, held under the left hand with tail between thumb and forefinger, and clipped to the tail by an appropriately shaped handle, thus securing it in place. Insemination was performed with a blunt-pointed 19 gauge needle and tuberculin syringe. With the aid of light from a head mirror the needle was inserted first into one horn of the uterus and then into the other, the point of the needle being turned towards the horn which it was desired to enter. Approximately 0.04 cc. of warm sperm suspension (1,000,000–2,000,000 sperm) were injected into each horn. The female was then placed with a sterile-tested vasectomized male and examined 1 to $1\frac{1}{2}$ hours later for a plug. If a plug was found, the female was killed several days later and the embryos counted. Of fifty-two inseminations, thirty-three failed to give plugs, three gave plugs after $1\frac{1}{2}$ hours and were sterile, three gave plugs within $1\frac{1}{2}$ hours and were sterile, thirteen gave plugs within $1\frac{1}{2}$ hours and were normally fertile.

SNELL, G. D., see Murray et al.

STEINBERG, A. G., see Fraser

STULLKEN, et al. ANOXIC SURVIVAL OF MICE AND CHICKS IN DECOMPRESSED ATMOSPHERES OF AIR AND OF OXYGEN. D. E. STULLKEN AND WM. A. HIESTAND, Purdue University.

Adult white mice averaging 17 g. weight were submitted to gradually falling barometric pressures until dead. The rate of decompression averaged 114 mm.

Hg. per minute. During decompression air (or oxygen) was admitted to the decompression chamber slowly by an inlet valve. When decompressed in oxygen an ample amount of oxygen (14 volumes) was passed through the chamber to wash out all possible air and a constant stream continuously admitted. The average barometric pressure at death in decompressed air for mice was 158.6 mm. Hg.; at death in decompressed oxygen was 70.7 mm. Hg. The same procedure was followed with baby chicks which died at an average barometric pressure of 218.3 mm. Hg. in air and of 89.5 mm. Hg. in oxygen.

Thus a considerable species difference exists in hypoxic resistance of chicks and mice. Since chicks are more physiologically independent at birth than are mice it would naturally be expected that they are less resistant to hypoxia than are mice. Since the mice used were adults and their known hypoxic resistance is less than that of young mice the species difference is even more apparent.

A comparison of the pO_2 at death in air and in oxygen indicates that alveolar pH_2O and/or pCO_2 must be quite low as compared with man. Mice probably have a higher lethal altitude ceiling than do humans due in part no doubt to the high rate of hyperventilation of the mouse.

STULLKEN, D. E., see Hiestand et al. 1 and 2

TABER, et al. OBSERVATIONS ON ERYTHROCYTE NUMBER IN BROWN LEGHORNS FOLLOWING INJECTIONS AND PELLET IMPLANTATIONS OF TESTOSTERONE PROPIONATE. Elsie Taber and L. V. Domm, Whitman Laboratory of Experimental Zoology, The University of Chicago.

We have shown that testosterone propionate raises erythrocyte counts of capons and hens to or beyond male level. To determine the period at which this effect first becomes apparent and the length of time it persists following cessation of injections, four capons were given 1 mg. testosterone propionate daily for 72 days. Counts were made every 2 or 4 days. On 10th day counts were significantly above four controls. By 20th day counts reached male level, remaining there throughout course of injections and for 16 days thereafter. By 28th day counts had returned to control level.

Counts were made on four 5-month-old pullets implanted with testosterone propionate pellets, averaging 34.8 mg. On 16th day counts reached male level, which was maintained 24 days. Counts returned to control level by 58th day. A similar rise and fall were observed after each of two successive implantations. Results indicate that male erythrocyte level is obtained in capons or pullets within 2 to 3 weeks by injections or implantation of testosterone propionate, and that there is considerable lag in returning to control level after treatments cease.

Six 1-month-old pullets received pellets averaging 14.3 mg. Although comb growth was excellent, there was no increase in erythrocytes. Ten days after a second implant (averaging 26.8 mg.) on 109th day, counts began to rise. This seems to suggest that the absorption rate from small pellets is beneath the threshold for erythrocyte response or that young pullets are incapable of response. Observations in progress may clarify these points.

TAHMISIAN, T. W., see Bodine et al.

TREAT. THE CHRONIC SPINAL ANIMAL. Asher E. Treat, College of Physicians and Surgeons, Columbia University and College of the City of New York. (Introduced by P. L. Bailey, Jr.)

A motion picture film (16 mm.) showing at normal speed and in slow motion, the reflexes exhibited by a cat which survived more than 26 months following surgical transection of the cervical spinal cord. Details of the care of a spinal preparation are shown. Near the conclusion of the film there is seen a reflex convulsion or "mass reflex", which, so far as is known, has not previously been photographed in the spinal cat.

VILLEE. PHENOGENETIC STUDIES OF THE MUTANT BITHORAX-34E OF *DROSOPHILA MELANOGASTER*. Claude A. Villee, University of North Carolina.

Bithorax is a homoeotic mutant which changes the metathorax and haltere more or less into a mesothorax and wing. The various phenotypes were studied and classified in a series ranging from a normal haltere (type 1) to a large, flat, wing-shaped structure complete with four longitudinal veins (type 7). These parallel the series described by Astauroff ('29) for tetraptera. As in many other homoeotic mutants, the expression of bithorax may be altered by exposing developing larvae to cold (15°C.) or heat (29°C.). Bithorax flies raised throughout development at 29°C. showed a slight but definite increase in the expression of the mutant over the controls. Heat treatments begun 0 to 4 days after eggs were laid and continued 1 to 6 days all produced some increase in expression, shifting it toward the wing condition. Cold treatments begun 0 to 5 days after eggs were laid and continued 1 to 8 days all decreased the expression, shifting it toward the haltere condition. The decrease was more or less proportional to the length of the treatment. Treatments begun 3 or 4 days after egg deposition produced the greatest decreases in expression. Neither cold nor heat showed a definite Temperature Effective Period for the reaction involved. These results are in line with previous experiments with tetraltera, in which heat treatments shifted the development of the mesothoracic disc toward the wing phenotype and cold treatments shifted development toward a haltere phenotype.

WALTON. THE PARASITES OF THE AMPHIUMIDAE (AMPHIBIA — CAUDATA). A. C. Walton, Knox College, Galesburg, Illinois.

Annotated lists of the parasites of the Amphiumidae indicate the following hosts and parasites: (a) *Amphiuma means* (U.S.A.) — *Porrocaecum amphiumae* (Nematoda); and *Cephalogonimus amphiumae*, *Crepidostomum cornutum*, *Megalodiscus amercianus*, and *Telorchis stunkardi* (Trematoda). (b) *Amphiuma tridactylum* (U.S.A.) — *Agamascaris odontocephala*, *Icosiella quadrituberculata*, *Ophidascaris labiatopapillosa*, and *Spirogonura cryptobranchi* (Nematoda); *Brachycoelium salamandrae*, *Cephalogonimus amphiumae*, larval *Clinostomum* sp?, *Halipegus fusiporus*, larval *Lechriorchis* sp?, *Megalodiscus americanus*, *Telorchis reelfooti*, and *T. stunkardi* (Trematoda); *Crepidobothrium amphiumae*, *Cysticercus* sp?, and *Ophiotaenia* sp? (Cestoda); and *Acanthocephalus* sp? (*Acanthocephala*).

WALTON. THE PARASITES OF THE PLETHODONTIDAE (AMPHIBIA—CAUDATA). I.
A. C. Walton, Knox College, Galesburg, Illinois.

Annotated lists of the parasites of the Plethodontidae indicate the following hosts and parasites: (a) *Aneides lugubris* (U.S.A.) — *Hexamita batrachorum* and *H. ovata* (Protozoa). (b) *Batrachoseps attenuatus* (U.S.A.) — *Haemogregarina riedyi*, *Hexamita batrachorum*, *H. ovata*, *Karotomorpha swezyi*, *Proteromonas longifila*, and *Trichomitus parvus* (Protozoa). (c) *Ensatina eschscholtzii* (U.S.A.) — *Hexamita batrachorum*, *H. ovata*, and *Monocercomonoides melolonthae* (Protozoa). (d) *Eurycea bislineata bislineata* (U.S.A.) — *Cystidicola* sp?, and *Omei chickasawi* (Nematoda); and *Brachycoelium salamandrae* (Trematoda). (e) *Eurycea bislineata cirrigera* (U.S.A.) — *Brachycoelium salamandrae* (Trematoda); proteocephalid cysts (Cestoda); *Cepiedietta fimbriata*, *Haptophrya michiganensis*, *Proteromonas longifila*, and *Trichomonas augusta* (Protozoa); and *Hannemania dunni* (Acarina). (f) *Eurycea bislineata wilderae* (U.S.A.) — “*Oxyuris*” *magnavulvaris* (Nematoda); *Brachycoelium salamandrae* (Trematoda); proteocephalid cysts (Cestoda); and *Cytamoeba bacterifera*, *Eutrichomastix batrachorum*, *Haptophrya michiganensis*, *Hexamastix batrachorum*, *Hexamita batrachorum*, *Proteromonas longifila*, and *Trichomonas augusta* (Protozoa). (g) *Eurycea longicauda* (U.S.A.) — *Karotomorpha swezyi* (Protozoa). (h) *Eurycea longicauda gutto-lineata* (U.S.A.) — *Cystidicola stigmatura*, *Oswaldocruzia euryceae*, and “*Oxyuris*” *magnavulvaris* (Nematoda); *Brachycoelium salamandrae*, *Gorgoderina tenua*, and *Plagitura salamandra* (Trematoda); proteocephalid cysts (Cestoda); *Cryptobia borrelli*, *Cytamoeba bacterifera*, *Eutrichomastix batrachorum*, *Haptophrya michiganensis*, *Hexamastix batrachorum*, *Hexamita batrachorum*, *Proteromonas longifila*, and *Trichomonas augusta* (Protozoa); and *Hannemania dunni* (Acarina). (i) *Gyrinophilus porphyriticus* (U.S.A.) — *Gorgoderia cygnoides* (Trematoda). (j) *Gyrinophilus porphyriticus danieli* (U.S.A.) — *Omeia papillocauda* (Nematoda); and *Hexamastix batrachorum* and *Proteromonas longifila* (Protozoa). (k) *Hemidactylum scutatum* (U.S.A.) — *Brachycoelium salamandrae* (Trematoda); and *Haptophrya michiganensis* (Protozoa).

WALTON. THE PARASITES OF THE PLETHODONTIDAE (AMPHIBIA—CAUDATA) II.
A. C. Walton, Knox College, Galesburg, Illinois.

Annotated lists of the parasites of the Plethodontidae indicate the following hosts and parasites: (a) *Desmognathus fuscus* (U.S.A.) — *Angiostoma plethodontis*, *Omeia papillocauda*, “*Oxyuris*” *magnavulvaris*, *O. papillocauda*, *Oswaldocruzia leidy*, *Physaloptera* spp?, and *Rhabdias entomelas* (Nematoda); *Brachycoelium salamandrae*, *B. sp?*, *Diplostomulum desmognathi*, *Gorgoderina bilobata*, *Megalodiscus temperatus*, various metacercariae, and *Phyllodistomum solidum* (Trematoda); *Ophiotaenia cryptobranchi*, and proteocephalid cysts (Cestoda); *Acanthocephalus acutulus* (Acanthocephala); *Cryptobia borrelli*, *Cytamoeba bacterifera*, *Eutrichomastix batrachorum*, *Haptophrya michiganensis*, *Hexamastix batrachorum*, *Hexamita batrachorum*, *H. intestinalis*, *Karotomorpha swezyi*, and *Proteromonas longifila* (Protozoa); and *Hannemania dunni* (Acarina). (b) *Desmognathus fuscus auriculatus* (U.S.A.) — *Brachycoelium salamandrae* (Trematoda).

toda). (c) *Desmognathus ochrophaeus* (U.S.A.) — *Proteromonas longifila*, and *Trichomonas augusta* (Protozoa). (d) *Desmognathus ochrophaeus carolinensis* (U.S.A.) — (*Capillaria inequalis*, and "Oxyuris" *magnavulvaris* (Nematoda); *Brachycoelium salamandrae* (Trematoda); *Ophiotaenia cryptobranchi* (Cestoda); and *Cryptobia borrelli*, *Eutrichomastix batrachorum*, *Hexamastix batrachorum*, *Hexamita intestinalis*, *Karotomorpha swezyi*, *Proteromonas longifila*, and *Trichomonas augusta* (Protozoa). (e) *Desmognathus phoca* (U.S.A.) — *Omeia papillocauda*, "Oxyuris" *magnavulvaris*, and spirurid cysts (Nematoda); *Brachycoelium salamandrae*, and *Diplostomulum desmognathi* (Trematoda); larval *Crepidobothrium* sp?, and *Ophiotaenia cryptobranchi* (Cestoda); and *Cryptobia borrelli*, *Cytamoeba bacterifera*, *Eutrichomastix batrachorum*, *Haptophrya michiganensis*, *Hexamastix batrachorum*, *Hexamita batrachorum*, *H. intestinalis*, *Karotomorpha swezyi*, *Proteromonas longifila*, and *Trichomonas augusta* (Protozoa). (f) *Desmognathus quadramaculatus* (U.S.A.) — *Capillaria inequalis*, *Omeia papillocauda*, "Oxyuris" *magnavulvaris*, and spirurid cysts (Nematoda); *Brachycoelium salamandrae*, *Diplostomulum desmognathi*, and various metacercariae (Trematoda); larval *Crepidobothrium* sp?, *Ophiotaenia cryptobranchi*, and proteocephalid cysts (Cestoda); *Acanthocephalus* sp? (*Acanthocephala*); and *Cryptobia borrelli*, *Cytamoeba bacterifera*, *Eutrichomastix batrachorum*, *Hexamastix batrachorum*, *Hexamita batrachorum*, *H. intestinalis*, *Karotomorpha swezyi*, *Proteromonas longifila*, and *Trichomonas augusta* (Protozoa). (g) *Desmognathus* spp? (U.S.A.) — *Oligobdella biannulata* (Hirudinea).

WANG, H., see Lillie et al.

WEISSENBERG. FINER STRUCTURES IN THE INCLUSION BODIES OF THE LYMPHO-CYSTIS VIRUS DISEASE OF FISH. J. Richard Weissenberg, Department of Histology, Middlesex University School of Medicine.

In the lymphocystis virus disease of fish the invaded host cells, represented by hypertrophied fibroblasts of gigantic size, contain characteristic cytoplasmic inclusions. These consist of a framework which stains like basichromatin and a ground substance which shows acidophilic staining reaction in *Pleuronectes*. With magnification of 1000 times or more the membranelles of the framework sometimes appear stippled which structure under favorable optical conditions (thin sections, appropriate differentiation of the staining, surface view of the membranelle) can be resolved into a layer of fine basophil granules. Such observations have been made in several preparations of lymphocystis inclusions of *Lepomis gibbosus*, *Acerina cernua*, *Stizostedion vitreum*. The basophil granules have been rather constantly found in the inclusions of young lymphocystis cells of *Pleuronectes flesus* (stage of the 68th day of the infection experiment) after fixation with acid fixatives.

Whereas I interpreted in 1921 the basophil framework of the inclusions as a reaction product of the host cell and supposed that only the granular ground substance might contain the virus units, I think it now more probable that the basophil framework represents virus substance. This view is supported by the fact that nucleoproteins have been found as components in the chemical analysis

of all viruses studied hitherto in a purified state. That the basophil substance of the lymphocystis inclusions contains nucleoprotein of the thymonucleic acid type is proved by its elective staining with methyl green as well as by its positive Feulgen reaction, as stated by Jirovec ('32).

WICHTERMAN. CONJUGATION AND FATE OF EXCONJUGANTS OF ZOÖCHLORELLA-FREE *PARAMECIUM BURSARIA*.¹ Ralph Wichterman, Temple University.

When organisms from a clone of *P. bursaria* (B 7) were mixed with individuals from a clone of Zoöchlorella-free *P. bursaria* (B 9) the mating reaction occurred with subsequent pairing for conjugation 24-36 hours later. On clear days, the organisms came together immediately between 11:30 A. M. and 1:30 P. M. Occasionally long chains of individuals were formed prior to conjugation. To determine the fate of exconjugants resulting from mating the "green" B 7 individuals with specimens from the "white" B 9 clone, seven different mass matings were made over a period of 83 days. Conjugants were isolated and placed in glass depression plates containing desiccated lettuce infusion. Observations and records were made of fifty-seven isolated pairs of conjugants and their 114 exconjugants. A single organism not involved in conjugation was placed in each depression plate as a control. The majority of conjugants remained together for 48 hours. After conjugation, the exconjugants were isolated and fission rates obtained for approximately 15 days for each exconjugant. It was found that 82.5% of the exconjugants failed to divide after conjugation. With only two exceptions, when a green exconjugant divided, its former white mate also divided.

It is noteworthy that no zoöchlorellae passed from the green conjugant to the white during the time that the individuals remained together.

When seven different mass matings were made over a period of 56 days of normal green specimens of *P. bursaria* from another source, the results were strikingly different. From these mass matings, fifty-one pairs of conjugants were isolated as well as their 102 exconjugants. Only 14.4% of these exconjugants failed to divide after conjugation.

Average temperature for all observations was 20°C.

¹ This investigation was aided by a grant from the Committee on Research and Publication, Temple University.

WITSCHI, E., see Graves

WITSCHI. MINIMAL GYNOGENIC AND ANDROGENIC DOSAGES OF EIGHT STEROIDS AND STILBESTROL IN CASTRATED SPARROWS AND STARLINGS. Emil Witschi, The State University of Iowa.

Oil solutions of eight steroids and stilbestrol were injected intramuscularly in order to test their gynogenic and androgenic threshold values in the sparrow and the starling. Progesterone and desoxycorticosterone produce no apparent effects on sexual characters, even if given in very high dosages; in combination with estrone large dosages of progesterone diminish the response of the oviducts. The typical gynogens do not stimulate development of male sex characters; the approxi-

mate minimal daily amounts in gammas, producing a distinct enlargement of the oviducts within 10 days are as follows (values for starlings in parentheses): Estradiol 0.05 (0.1), estrone 0.2 (1), estriol 5 (20), and stilbestrol 0.2 (1). The threshold values for the stimulation of the vasa deferentia by androgens are higher, namely: testosterone propionate 5 (20), androsterone 20 (100), and dehydroandrosterone 60. The bills are twenty times more sensitive and their color reactions indicate the presence of testosterone in amounts equally small as those listed above for the oviduct-estrone threshold. It is well known that in all vertebrates, with the exception of the Eutheria, the androgens are also gynogenic, if administered in high quantities. In our birds the minimal daily doses producing oviduct stimulation were as follows: testosterone propionate 60 (200), androsterone 200 (600), and dehydroandrosterone 600. If simultaneously injected, testosterone adds its effect to that of estrone in the stimulation of the oviducts, but estrone partially inhibits the response of the vasa deferentia to testosterone.

WORLEY. THE ORIGIN OF THE GOLGI SUBSTANCES DURING OVOGENESIS. Leonard G. Worley, Brooklyn College.

The number of Golgi bodies — granules and droplets — that make their appearance in the early oocytes of many animals is too great to be accounted for as the result of the multiplication of the few pre-existing Golgi elements, especially since there is little indication that any multiplication is occurring during this period. Furthermore, these bodies are not believed to arise strictly "de novo". Practically all of the evidence at present indicates that in these early stages the Golgi bodies take their origin from formed protein elements in the cytoplasm which previously were discharged from the nucleus as nucleolar emission granules and droplets. Once outside the nucleus, these extrusions gradually develop into Golgi droplets very probably through the absorption of lipids from the cytoplasm. In this condition, the Golgi bodies are homogeneous in appearance, but are both fuchsinophilic and osmiophilic. Soon, however, coincident with enlargement, a segregation of the protein and lipoidal components takes place resulting in the typical vesicular Golgi body structure, i. e., lipoidal, osmiophilic cortex and protein, fuchsinophilic, osmiophobic core. These observations may account, in part, for the well known confusion on the question as to whether the albuminous yolk arises from Golgi elements or from nucleolar extrusions. The absorption of substances from the cytoplasm and the segregation of protein and lipid appear to be two of the most characteristic features of Golgi body activity in many animal eggs.

WULFF, et al. CORRELATION OF E.M.F. AND PHOTOCHEMICAL CHANGES IN THE DARK-ADAPTED INSECT EYE. Verner John Wulff and Theodore Louis Jahn, The State University of Iowa.

The present paper is an attempt to correlate the magnitude of the E.M.F. of the electroretinogram with the predictable photochemical changes in the dark adapted insect eye. A similar correlation for the light adapted eye has been made previously (Wulff, '43).

If the voltage of the electrical response of grasshoppers and moths is plotted against the logarithm of the intensity of the stimulating light ($\log I$) a sigmoid curve is obtained. If the percent concentration of visual purple breakdown product (as determined experimentally by Hecht) is plotted against $\log I$ another sigmoid curve is obtained. Therefore, there seems to be a correlation between the E.M.F. and the probable concentration of photochemical product.

However, under the conditions of Hecht's experiments the reaction was irreversible, whereas under the conditions of the present experiments the reaction was reversible and the reverse reaction might have preceded to an appreciable extent. Therefore, it was necessary to compute the theoretical amount of photochemical product formed as a function of intensity with reversibility considered in the calculations. The equation for this computation has also been furnished by Hecht. Values of the percent concentration of photochemical product plotted against $\log I$ for this equation also yield a sigmoid curve.

Therefore, it seems as if there is a linear relationship between the E.M.F. of the electroretinogram and the amount of photochemical product. This is in harmony with the theory that the electrical change in the sense cell may be the agent which stimulates the optic nerve (Wulff, '43).

WULFF, V. J., see Jahn et al.

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